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THE INHIBITORY ACTION OF NICOTINE ON THE RABBIT COLON

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The response to nicotine of intestinal preparations *in vitro* is usually a contraction due to stimulation of the parasympathetic cholinergic neurones in Auerbach's plexus. Ambache (1951) and Ambache & Edwards (1951) found that an inhibitory effect of nicotine could be demonstrated if these intrinsic cholinergic motor neurones were first put out of action, either by *Clostridium botulinum* toxin (rabbit small intestine) or by atropine (kitten small intestine and stomach). This effect could be blocked by nicotine in ganglion-cell-paralysing doses and by hexamethonium. In the ileum of the rabbit an inhibitory effect of nicotine could not be produced in the presence of atropine because the motor effects of nicotine were not abolished by atropine unless high, and probably non-specific, concentrations of this drug were used. In contrast we have found previously, in experiments on preparations of the rabbit colon, that nicotine could produce inhibition in this region of the alimentary canal. By selecting suitable dose levels of nicotine we could obtain either pure inhibitory responses (with low doses), or pure motor responses (with high doses), or mixed inhibition and contraction; cholinergic blocking agents were not required in these experiments.

Nicotine is known to produce in other organs peripheral effects which are apparently due to the release of catechol amines, in particular, acceleration of the heart (Hoffman, Hoffman, Middleton & Talesnik, 1945; Kottegoda, 1953*a*), constriction of blood vessels (Kottegoda, 1953*b*; Hilton, 1954; Burn & Rand, 1958; Strömblad, 1959) and dilatation of bronchial smooth muscle (Hawkins & Paton, 1958). Ambache (1951) attributed the inhibition with nicotine in the intestine to a release of catechol amines from the nerve endings of adrenergic neurones in Auerbach's plexus, since it could be blocked by ephedrine. In other tissues chromaffin cells have been suggested as an alternative source of the catechol amines. Since nicotine-induced inhibition can be demonstrated easily in the colon, we have used this preparation for further investigations of the mechanism of nicotine action and, in particular, to decide whether chromaffin cells, rather than adrenergic neurones, might be the source of

the inhibitory amines. The results do not support the view that chromaffin cells are responsible for this effect.

METHODS

Rabbits of either sex were killed by a blow on the neck and bled. Preparations were made, as described by Garry & Gillespie (1955), of the rabbit colon complete with its extrinsic sympathetic (lumbar colonic) and parasympathetic (pelvic) nerve supplies, each of which could be stimulated separately. Magnus preparations of colon and ileum, without dissected lengths of the extrinsic nerves, were also used. The preparations were suspended in Krebs's saline solution at 36° C in a 200 ml. isolated organ bath, and the contractions of the longitudinal muscle were recorded on a smoked drum with a light gimbal isotonic lever (magnification 3 ×); the tension on the preparations was 0·5 g. The composition of the bath fluid was as follows: (g/l.) NaCl, 6·92; KCl, 0·35; $CaCl_2$, 0·28; KH_2PO_4, 0·16; $MgSO_4.7H_2O$, 0·29; $NaHCO_3$, 2·1; glucose, 2.

To make mucosa-free preparations the colon was first converted into a flat strip by a longitudinal cut along the antimesenteric border, and the preparation was pinned out on cork, mucosal side upwards. The line of cleavage between the submucosa and the underlying muscle layer was easily identified and the mucosa and submucosa were stripped off with fine forceps, preferably in the direction of the circular muscle. When suspended in the organ bath such preparations displayed the rhythmic activity usual in normal preparations, with the added advantage of a more stable base line; they responded well to electrical stimulation of the extrinsic sympathetic and parasympathetic nerves.

Two methods were used for staining the chromaffin cells in the gut. First, the chromaffin reaction, unmodified. The piece of intestine was opened out, pinned flat on to a cork board with quills, and then fixed for 24 hr in Müller's fluid containing 10% formalin. The material was then 'post-chromed' for 3 days in a 2·5% aqueous solution of potassium bichromate, after which it was washed, dehydrated, embedded in paraffin and sectioned at 7 μ. The sections were subsequently lightly counter-stained with haemalum. The second method was that of Sevki, as described by Adams-Ray & Nordenstam (1956). The preliminary treatment was the same as above but, after embedding in paraffin, the tissue was sectioned and the sections stained by a modified Giemsa method.

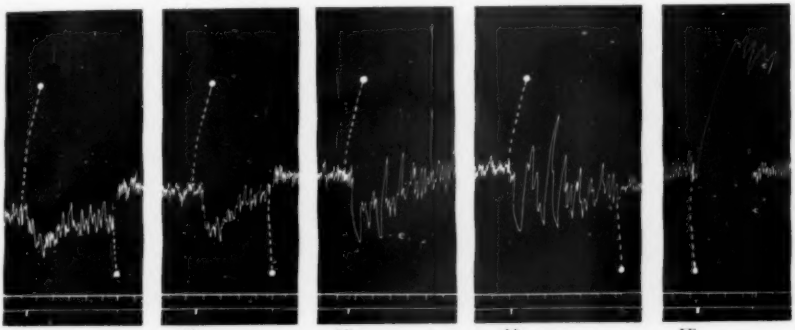
The following drugs were used: adrenaline hydrochloride, acetylcholine chloride, atropine sulphate, dimethylphenyl-piperazinium iodide (DMPP), hexamethonium bromide (May & Baker), nicotine hydrogen tartrate, reserpine (Serpasil; Ciba), and choline 2:6 xylyl ether bromide (TM 10). Concentrations refer to the salts.

RESULTS

The action of nicotine and DMPP. It is well known that the response to nicotine of the rabbit's small intestine is always a contraction. In the colon the type of response varies with the concentration of nicotine. With low concentrations the first response is always inhibition. When the concentration of nicotine is increased the response becomes first biphasic, with inhibition followed by contraction, and finally purely motor. These results are shown in Text-fig. 1. The concentration of nicotine which will produce pure inhibition varies from one preparation to another, but is usually between 10^{-6} and 10^{-5}. As Ambache & Lessin (1955) have shown, in the ileum the ganglion-stimulating drug DMPP can also cause

inhibition. In the colon the effect is neither greater nor more readily elicited than with nicotine and the drug was not used extensively.

Effect of atropine. Nicotine in concentrations greater than 10^{-5} causes the colon to contract. It is possible that in these concentrations it also stimulates the inhibitory mechanism, but this is masked by the strong contraction arising from the stimulation of the cholinergic ganglion cells in Auerbach's plexus. If the effects of stimulation of these motor neurones could be blocked at the nerve–muscle junction by atropine, then it should be possible to reverse the normal motor response by uncovering the co-existing inhibition. This expectation was fulfilled, as is shown in Text-fig. 2. The response at *A* to nicotine 10^{-5} before atropine was a contrac-

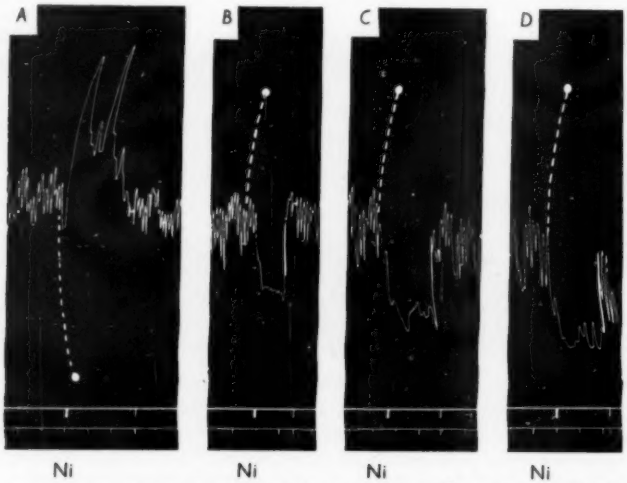


Text-fig. 1. Rabbit colon preparation (*in vitro* in this, as in all subsequent experiments). Curved dotted lines indicate addition of nicotine (Ni), or washing. Nicotine in low concentrations elicits an inhibitory, and in high concentrations a motor, response. In all text-figures time marker = 30 sec.

tion; at *B* in the presence of atropine 10^{-4}, nicotine 10^{-5} produced inhibition. The concentration of atropine used was that which was necessary just to block the response to stimulation of the parasympathetic (pelvic) nerves, on the assumption that these nerves have as their final pathway the same motor neurones as are stimulated by nicotine. Atropine in such high concentrations, besides blocking 'muscarinic' effects, also reduced 'nicotinic' effects slightly (Text-fig. 2). Thus, small doses of nicotine, which before atropine produced inhibition, were ineffective after atropine. However, larger doses of nicotine, previously eliciting contractions, now had a reverse effect, i.e. inhibition. Fortunately, after the atropine was washed out, its anti-muscarinic blocking action persisted for some hours, as was shown by the block of the motor response to pelvic nerve stimulation. In contrast, the antagonism by atropine of nicotine inhibition disappeared fairly quickly, as shown for example in Text-fig. 2 by the

increasing effectiveness of nicotine in producing inhibition, 20 min and 40 min respectively after the removal of atropine.

In most experiments atropine was used in this way to demonstrate the inhibitory effect of nicotine. Atropine sulphate 10^{-4} or 2×10^{-4} was added to the bath fluid and left in contact with the preparation until the response to stimulation of the pelvic nerve was blocked. The atropine was then removed and nicotine in a concentration of 10^{-5} added, to produce inhibition. This method reliably demonstrated inhibition and avoided the necessity of determining in each preparation the exact dose of nicotine, which would, acting alone, produce inhibition.



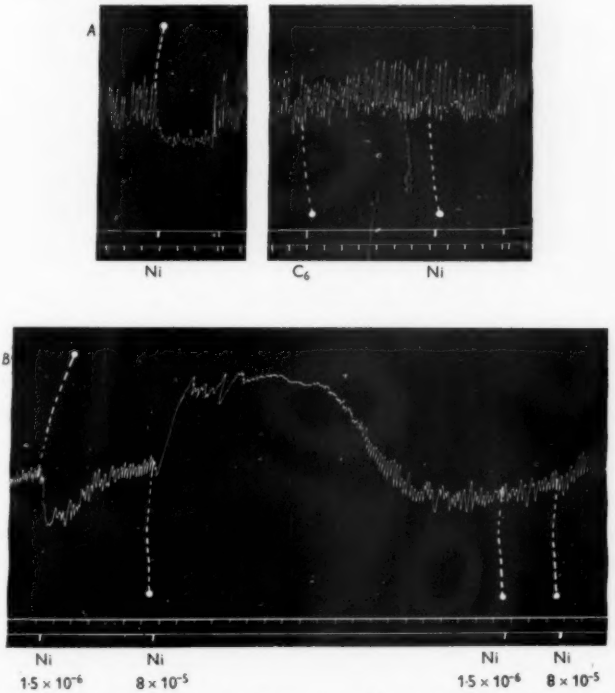
Text-fig. 2. Reversal by atropine of the nicotine response in the rabbit colon. At Ni, nicotine 10^{-5}. *A*, before atropine; *B*, in the presence of atropine 10^{-4}; *C* and *D*, 20 and 40 min, respectively, after washing out the atropine.

Effect of hexamethonium and of large doses of nicotine. Ambache (1951) and Ambache & Edwards (1951) found that the inhibitory action of nicotine was abolished by hexamethonium or by ganglion-paralysing doses of nicotine. In our experiments too, hexamethonium bromide added to the bath in concentrations similar to those required to block the response to stimulation of the pelvic nerve, also blocked the inhibitory effect of nicotine (Text-fig. 3*A*). Although high concentrations of hexamethonium were required, its action could be shown to be specifically ganglion-blocking in that the response to stimulation of the lumbar sympathetic nerves (Text-fig. 6) and the responses to administered acetylcholine or noradrenaline were unaltered.

If large ganglion-paralysing doses of nicotine were added to the bath

and left in contact with the preparation until the tone and activity had returned to their previous level, then the addition of further small doses of nicotine, which had previously caused inhibition, was now ineffective (Text-fig. 3*B*).

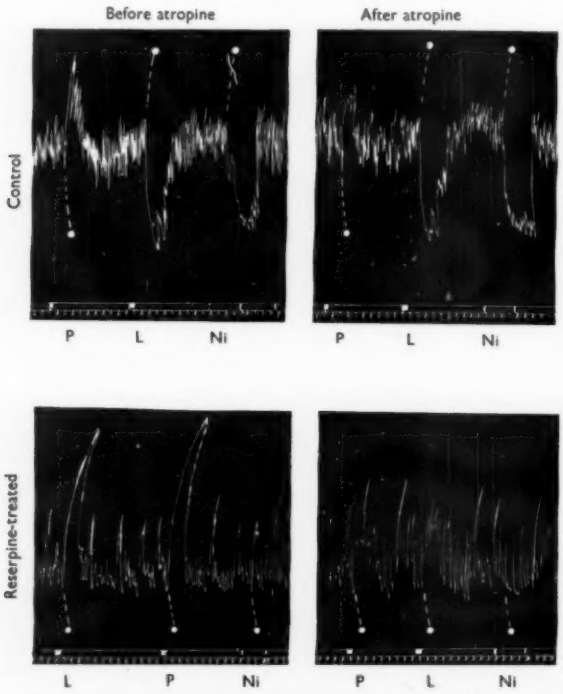
Effect of reserpine. The similarity between the inhibition produced by nicotine and the inhibition which follows stimulation of the sympathetic



Text-fig. 3. *A*. Extinction by 2×10^{-4} hexamethonium bromide (C_6) of the inhibition produced by nicotine 10^{-5} (Ni) in the rabbit colon. *B*. A high concentration of nicotine (8×10^{-5}) left in contact with the preparation abolishes both the inhibitory effect of a small dose of nicotine and the motor effect of a second, larger, dose of nicotine.

lumbar nerves suggested that the nicotine response might be mediated by the release of a catechol amine at some site in the gut wall. It is known that reserpine can produce a depletion of these amines at peripheral adrenergic nerve endings and, less readily, in chromaffin cells (Muscholl & Vogt, 1958); according to these authors' results it would seem that reserpine is more effective in repeated, small, doses, particularly on chromaffin cells. We have accordingly given rabbits single daily intravenous injections

of reserpine 0·2 mg/kg for 10 days. A group of eight animals was used, four treated with reserpine in a suitable vehicle and the other four, serving as controls, receiving daily injections of an equal volume of the vehicle by itself. In the four preparations from the reserpine-treated animals the



Text-fig. 4. Effect of reserpine on the response of the rabbit colon preparation to nicotine 10^{-5} (Ni) and to parasympathetic (P) and sympathetic (L) stimulation. Above, preparation from a control animal injected with the reserpine vehicle; below, from an animal given reserpine 0·2 mg/kg I.V. daily for 10 days. In the control, nicotine causes inhibition and this is enhanced after atropine. In the preparation from the reserpine-treated animal nicotine has little or no inhibitory action either before or after atropine, and the response to sympathetic nerve stimulation is reversed to motor.

inhibitory effect of nicotine was almost completely abolished, whereas in each of the four control animals powerful inhibitory responses were obtained (Text-fig. 4). An unusual finding in these experiments was that after reserpine depletion of the adrenergic nerve endings the response to sympathetic nerve stimulation not only ceased to be inhibitory but was, in fact, replaced by a motor response. This sympathetic reversal has been further investigated and will be reported separately.

Effect of choline 2:6 *xylyl ether* (*TM* 10). The results so far described have shown that the inhibition produced by nicotine in the rabbit colon was a true nicotinic effect of the drug, being blocked by hexamethonium and by large paralysing doses of nicotine, and that the action was mediated by the release of a catechol amine. There remained the problem of the site of liberation of this amine. The two obvious possibilities were (1) adrenergic neurones in Auerbach's plexus and (2) chromaffin cells. The following experiments were designed to distinguish between these two possibilities.

TM 10, although possessing numerous other actions, is known to block the effects of adrenergic nerve stimulation, apparently by interference with the synthesis or release of the transmitter (Exley, 1957). Bain & Fielden (1956) found that it abolished the effect of sympathetic nerve stimulation in the small intestine. Chromaffin cells, as for example in the adrenal medulla, are relatively resistant to the action of TM 10 and no effect on their release of catechols could be shown in acute experiments (Exley, 1957). This drug might therefore be expected to discriminate between an action of nicotine on the adrenergic neurones and on chromaffin cells.

In the present experiments on colon preparations, the response to stimulation of the adrenergic supply in the extrinsic sympathetic nerves was abolished after about 70 min exposure to TM 10. On the other hand, the inhibition produced by nicotine was only slightly reduced (Text-fig. 5) and the motor response to parasympathetic stimulation was also unaffected.

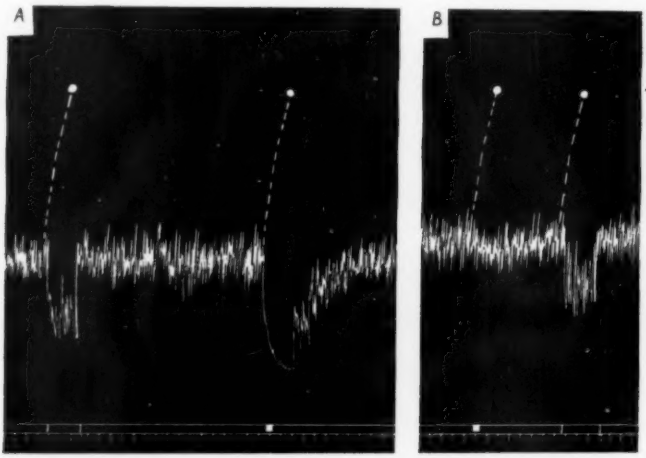
Histological findings. The above results suggested that the action of the nicotine was perhaps on chromaffin cells. We have therefore examined serial sections of lengths of colon for the presence of chromaffin cells or chromaffin tissue. As controls, lengths of colon from rabbits treated with reserpine were similarly examined after the reserpine had discharged the contents of the chromaffin cells. Other tissues examined were: stomach, duodenum, ileum, inferior mesenteric ganglion, and solar ganglion.

In the normal colon the only cells giving the chromaffin reaction were the enterochromaffin cells in the mucosa. The region of Auerbach's plexus was examined particularly carefully in a large number of sections without discovering any chromaffin-staining cells. The enterochromaffin cells also stained well with Sevki's method, assuming a dark reddish-brown or buff colour. In animals reserpine-treated for 5–10 days enterochromaffin cells disappeared completely from the colon, which was examined by both methods of staining.

With Sevki staining one other type of cell was found in the mucosa and submucosa of all parts of the alimentary tract examined (Pl. 1, *g*). They were much commoner in the small intestine than in the colon. These cells contained bright red, discrete granules: the cell outline was ill-defined, the granules often appearing to lie almost free; the cytoplasm was unstained and the disposition of the granules was very variable. The nucleus was commonly single, occasionally bilobed. These cells resembled the chromaffin cells in the skin illustrated

by Adams-Ray & Nordenstam (1956), but were not in fact chromaffin cells, since, in animals treated with reserpine, their staining properties remained unaltered, whereas the known enterochromaffin cells became invisible. From their bright red granules and occasional bilobed 'spectacle' nucleus it may be inferred that they are almost certainly eosinophils, a cell commonly found free in the gut wall. These cells together with typical enterochromaffin cells are illustrated in Pl. 1.

In the inferior mesenteric and solar ganglia, groups of typical chromaffin cells were found, chiefly on the outside of the ganglia, but occasionally single cells or small groups were found buried among the ganglion cells, as described by previous authors (Kohn, 1903; Muschol & Vogt, 1958).

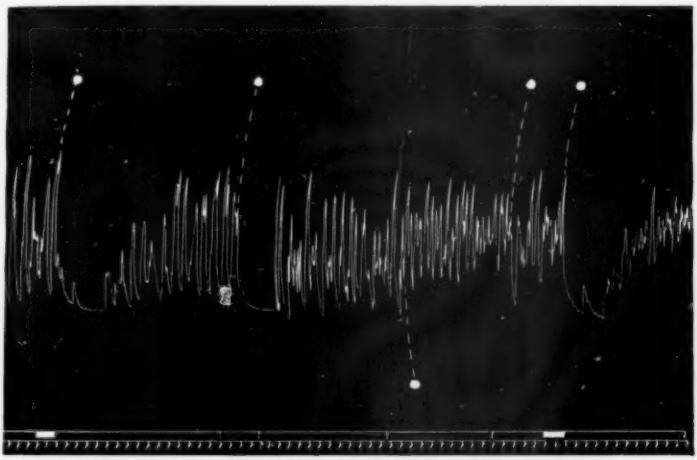


Text-fig. 5. Effect of TM 10 (choline 2:6 xylyl ether) on the response of the rabbit colon preparation to stimulation of the extrinsic sympathetic nerves (L) and to nicotine 10^{-5} (Ni). *A*, inhibitory responses produced initially by nerve stimulation and by nicotine. Between *A* and *B*, TM 10 (2×10^{-5}), abolishing inhibitory effect of sympathetic stimulation but not of nicotine, which is slightly reduced.

Chromaffin cells were not found in the stellate ganglion. It should be mentioned here that in the innervated colon preparation which we used for our nicotine experiments the inferior mesenteric ganglion is retained. Nicotine, however, could not have produced inhibition by liberating adrenaline or noradrenaline from this ganglion into the bath fluid, since even when all the mesentery and external nerves had been removed inhibition was still produced by nicotine. Thus Text-fig. 3*B* is from such a preparation.

Effect of mucosal stripping. The only chromaffin cells demonstrable in the gut wall were the enterochromaffin cells in the mucosa. It is almost certain that these cells contain 5-hydroxytryptamine (5-HT), a substance which

has a motor rather than an inhibitory effect on the smooth muscle of the colon. It was, however, conceivable that these cells might also produce catechol amines, either together with 5-HT in the same cell, or if the cell population were not uniform, in a few cells producing only catechol amines. If such catechol-amine-liberating cells were present, then removal of the mucosa ought to abolish the inhibitory effect of nicotine. After stripping the mucosa, the smooth muscle appeared undamaged since it continued to respond normally to stimulation of both sympathetic and



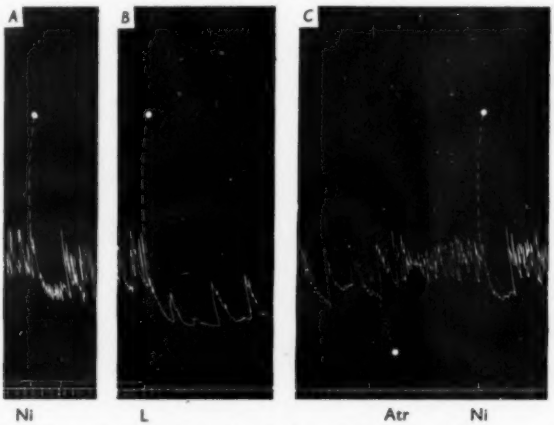
Text-fig. 6. Mucosa-free rabbit colon preparation, showing normal responses to sympathetic nerve stimulation (L) and to nicotine 10^{-5} (Ni). Hexamethonium (C_6) 10^{-4} abolishes the response to nicotine without affecting the response to sympathetic nerve stimulation.

parasympathetic nerves; moreover, the inhibitory response to nicotine was also unaffected, as shown in Text-fig. 6. In this experiment the inhibitory responses to lumbar nerve stimulation and to 10^{-5} nicotine were similar; but the nicotine response was completely blocked by hexamethonium, as before, whereas the response to stimulation of the post-ganglionic sympathetic nerves was unaffected.

Nerve fatigue and degeneration. The histological evidence that the only chromaffin cells present were those found in the mucosa, reinforced by the clear evidence that removal of these cells did not abolish the inhibitory effect of nicotine, redirected attention to the sympathetic nerves as the source of the catechol amines. The possibility that nicotine might release sympathin directly from sympathetic nerve endings was therefore considered. To test this, an attempt was made to reduce or to eliminate this sympathin in the gut wall by two methods. First, the nerves were

stimulated for long periods at high frequency (50/sec) until the preparation had 'escaped' from the initial inhibition; nicotine was then added while the stimulation was continued. The result is shown in Text-fig. 7. Even when the preparation had 'escaped' and recovered much of its original tone and rhythmic activity, i.e. when the amount of sympathin liberated from the nerve endings was presumably reduced, nicotine added to the bath still produced inhibition as before.

Secondly, the sympathetic nerves were cut and allowed to degenerate for 14 days. In these animals the cut peripheral ends of the nerves were stimulated during the subsequent experiment *in vitro*, to demonstrate the completeness of the denervation and degeneration. It was surprising how



Text-fig. 7. Effect of exhaustive stimulation of the sympathetic nerves on the inhibitory action of nicotine. *A*, response to nicotine 10^{-5} before nerve stimulation; *B*, the beginning of sympathetic stimulation; *C*, 100 min later there is some escape from the inhibitory effect; nicotine (after atropine) still produces inhibition of nerve stimulation, as before.

often some residual inhibitory effect was obtained in preparations in which the inferior mesenteric ganglion had been completely removed and both the inferior mesenteric artery and the colonic vein apparently stripped clean. This may be due to the survival of a proportion of intact post-ganglionic fibres, originating in groups of ganglion cells situated distal to the main ganglionic mass but along the course of the artery. In those preparations in which there was a residual response from stimulation of the extrinsic sympathetic nerves nicotine still elicited an inhibitory response. Our impression was that this was weaker than would be expected in normal preparations. In those preparations in which stimulation of the extrinsic sympathetic nerves was ineffective, nicotine when added to the bath produced either no inhibition or, more often, only a small inhibitory response.

DISCUSSION

We have demonstrated that nicotine on the colon of the rabbit causes inhibition of the smooth muscle. This effect is not due to paralysis of intrinsic motor neurones, since it appears at concentrations lower than are required for stimulation of these neurones. The inhibition is abolished by hexamethonium in the same concentration as is required to block post-ganglionic parasympathetic neurones; and the time course of the onset of block and the recovery after washing is similar. The inhibition is therefore a true 'nicotinic' effect. It is apparently due to the release of catechol amines from some site in the gut wall, since reserpine, which produces depletion of these amines, also abolishes the inhibitory effect of nicotine.

Two possible sources of these catechol amines would fit the experimental results, either adrenergic neurones in Auerbach's plexus or chromaffin cells in the gut. In further experiments objections have arisen to both. TM 10 could be shown to block the inhibitory effect of known adrenergic neurones (the extrinsic sympathetic nerves), without substantially affecting nicotine inhibition. Histologically chromaffin cells were present only in the mucosa, yet removal of the mucosa did not abolish the inhibitory action of nicotine. Finally, section and degeneration of the extrinsic sympathetic nerves abolished or reduced the inhibitory response, indicating that the site of liberation of the catechol amines was probably the post-ganglionic extrinsic sympathetic nerve endings or some structure associated with them.

Coon & Rothman (1940) reported fleeting pilo-erection after intradermal injection of acetylcholine or nicotine in man and in the cat. This response was unaffected by acute section of the nerve supplying the area, but was abolished after degeneration of the sympathetic nerves. These authors attributed the effect to an axon reflex in the terminals of peripheral sympathetic pilomotor nerves. Hilton (1954) described a similar effect in the terminals of sympathetic vasoconstrictor fibres. The inhibition of the colonic musculature produced by nicotine appears to be a similar phenomenon in that it is unaffected by acute sympathetic nerve section but is reduced or abolished by degenerative sympathetic section. It could therefore be attributed to an axon reflex in the sympathetic nerve endings. It is, however, difficult to visualize exactly how nicotine initiates this reflex in efferent fibres. Neither nicotine nor acetylcholine even in large concentrations appears able to stimulate nerve fibres (Lorente de Nó, 1944; Hodgkin, 1947), which would suggest that the drug must act on the nerve endings. However, such evidence as there is suggests that nicotine or acetylcholine is as ineffective at efferent nerve endings as on nerve fibres. Acetylcholine added to the fluid perfusing sympathetic ganglia, even in

high concentrations, failed to stimulate the preganglionic sympathetic nerve endings and to initiate action potentials in the preganglionic fibres (Brown & MacIntosh, 1939; Bronk, 1939). Because of these difficulties we would like to suggest the possibility that nicotine acts, not on the adrenergic nerves proper, but on some structure related to these nerves and intervening in some way between them and the effector cells. This structure would then be responsible for the spread of the response, producing its effects by the liberation of a catechol amine.

The histological picture of the final innervation of smooth muscle by the autonomic nervous system is still obscure and the subject of considerable difference of opinion. This mainly centres round the nature of those small cells first described by Cajal (1909) as 'neurones sympathiques interstitiels', which form a network or ground plexus throughout all the tissues innervated by the autonomic nervous system. If these interstitial cells are some form of small, modified ganglion cells, then they would provide a much likelier site of action for nicotine than either nerve fibres or efferent nerve endings. To postulate such a nerve network brings its own difficulties. For example, in the present experiments after section and degeneration of the extrinsic sympathetic nerves the inhibitory effect of nicotine is reduced or abolished. Either the sympathetic ground plexus must also have degenerated, which seems unlikely, or else the catechol amines in the ground plexus are depleted in the absence of the extrinsic sympathetic nerves. This would make the sympathetic ground plexus a structure in which noradrenaline is stored; such stores have been postulated by Burn & Rand (1958) in the neighbourhood of adrenergic nerve endings in the walls of blood vessels and are believed to derive their noradrenaline from these nerve endings.

The existence of such a plexus might also help to explain one remaining inconsistency in our experiments. If nicotine produces inhibition by releasing catechol amines from the extrinsic sympathetic nerves, why is this inhibition not abolished by TM 10 when the effect of nerve stimulation is abolished? If an intervening step between the sympathetic nerves and the effector muscle exists, then it is possible that TM 10 acts on the sympathetic nerves, either to block the synthesis or the release of transmitter, whereas nicotine acts directly on the sympathetic ground plexus to release its stored noradrenaline. An alternative explanation not involving a ground plexus is that TM 10 blocks the release of transmitter from the nerve endings by nerve action potentials but not by nicotine.

Section and subsequent degeneration of the sympathetic nerves to the colon for 14 days produced a variable reduction in the inhibitory effect of nicotine. In one experiment inhibition was completely absent, but in others it was present though reduced. The reason for this is not known.

The post-ganglionic neurones are not confined to the inferior mesenteric ganglion. The few situated more peripherally would remain intact with our denervation technique and may be responsible for the residual inhibitory effect of nicotine. Other possibilities are that the stores from which nicotine releases the transmitter can be replenished by circulating adrenaline, as suggested by Burn & Rand (1959), or that after denervation the smooth muscle acquires a sensitivity to nicotine, as suggested by Winbury (1959). The possibility that in some sites nicotine may act otherwise than through the extrinsic nerves is suggested by the findings of Ginzel & Kottegoda (1953). These workers found that nicotine caused stimulation of rabbit auricles and vasoconstriction of the blood vessels of the ear even after sympathetic denervation and, in the ear, after sensory denervation.

We have found no evidence that the inhibitory effect of nicotine can be mimicked by stimulation of the pelvic parasympathetic nerves. Ambache (1951) suggested that the adrenergic neurones to which he attributed the inhibitory effect in the ileum were inhibitory neurones within the peristaltic reflex arc and might also be innervated by vagal preganglionic fibres. The experimental basis for this vagal innervation was the report by McSwiney & Robson (1929) on the variable character of the response of the stomach to stimulation of the vagus. It is probable that these variations in response are due to a mixture of cholinergic and adrenergic fibres in the vagus (Harrison & McSwiney, 1936; Garry & Gillespie, 1955). Certainly in our experiments after washing out the atropine and waiting for its anti-nicotinic effect to wear off (as shown by the recovery of the inhibitory effect of nicotine) there was no inhibitory response on stimulating the pelvic nerve.

SUMMARY

1. Nicotine in low concentration (10^{-6}–10^{-5}) causes inhibition of preparations of the rabbit colon *in vitro*. In high concentrations ($> 10^{-5}$) it causes contraction. The mechanism of the inhibitory response has been studied.

2. The inhibitory effect of nicotine was abolished by hexamethonium in concentrations similar to those required for blocking the ganglion cells in the parasympathetic pathway. Large, paralysing doses of nicotine likewise blocked the response.

3. The inhibitory action of nicotine was enhanced after atropine. The concentrations of atropine used were high and had some anti-nicotinic action as well as their more usual anti-muscarinic effect. A method is described of overcoming this side effect.

4. After daily intravenous injections of reserpine for 10 days the inhibitory effect of nicotine virtually disappeared, presumably because of

the depletion of catechol amines by the reserpine. Nicotine therefore appears to produce inhibition by liberating a catechol amine.

5. Choline 2:6 xylyl ether bromide (TM 10) suppressed the inhibition produced by sympathetic nerve stimulation but did not abolish the inhibition produced by nicotine.

6. After degenerative section of the extrinsic sympathetic outflow the inhibitory effect of nicotine was reduced or lost.

7. Histologically the only chromaffin cells found in the colon were the enterochromaffin cells in the mucosa. Removal of the mucosa did not abolish the inhibition produced by nicotine. Thus the participation of mucosal chromaffin cells in this effect is excluded.

8. The inhibitory effect of nicotine, therefore, seems to be due to release of catechol amines either (1) from the extrinsic sympathetic nerves or (2) from some structure associated with them. It corresponds to the pilomotor response in the skin described by Coon & Rothman (1940) and attributed by them to an axon reflex in efferent adrenergic fibres. The possibility that nicotine acts on some form of terminal sympathetic nerve network intervening between the sympathetic nerves and the smooth muscle is tentatively suggested.

We are indebted to Professor W. A. Bain for the supply of TM 10; to Messrs May and Baker and Ciba for hexamethonium bromide and reserpine respectively; and to Miss G. Docherty, Miss A. Gilroy and Miss A. McCaffery for skilful technical assistance. The Rankin Medical Research Fund helped to provide apparatus.

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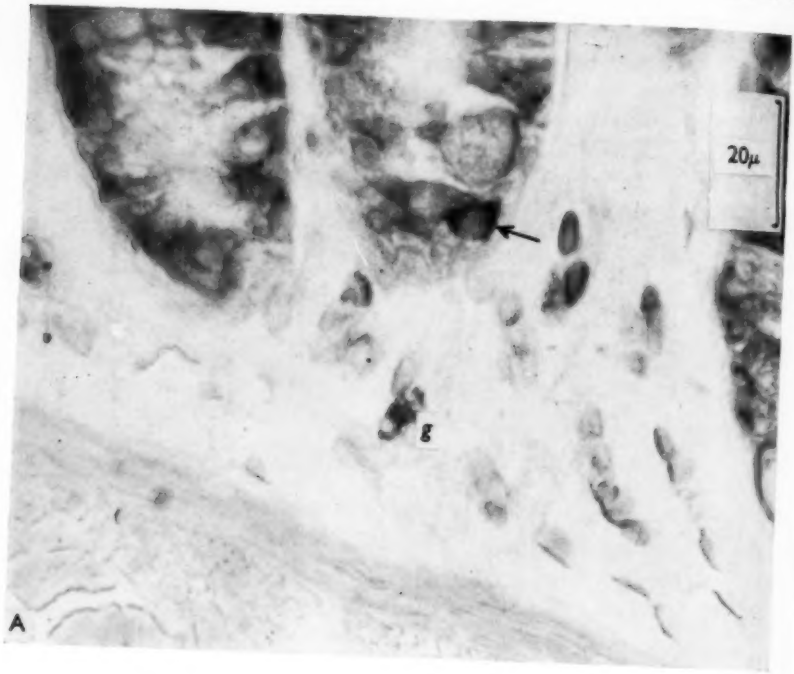

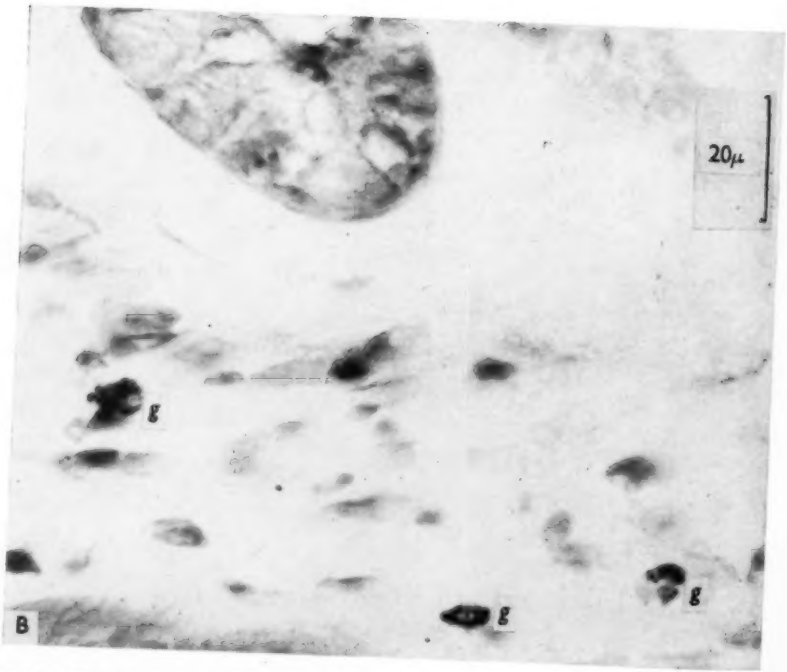

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EXPLANATION OF PLATE

PLATE 1

A. Section of normal rabbit colon stained by Sevki's method and showing an enterochromaffin cell (arrowed) in the mucosal glands and a granular cell (*g*) lying free in the stroma of the mucosa.

B. Section of the colon from a rabbit treated for 5 days with reserpine 0·2 mg/kg daily for 3 days and 1 mg/kg daily for a further 2 days. Enterochromaffin cells are absent, but the granular cells are unaltered.

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ACTIONS AND INTERACTIONS OF ALDOSTERONE MONO-ACETATE AND NEUROHYPOPHYSIAL HORMONES ON THE ISOLATED CAT KIDNEY

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Intravenous infusions of quantities of aldosterone too small to affect renal function raised the concentration of potassium in the plasma of cats under chloralose anaesthesia (Davey & Lockett, unpublished). This observation indicated extra-renal action of the hormone and raised the question whether the effects of aldosterone on renal function were predominantly the result of the direct action of this compound within the kidney itself or were, in large part, the consequence of its extra-renal effects. The existence of extra-renal actions of mineralocorticoids is well known; they have been described by Wilson (1957) and Flanagan, Davis & Overmann (1950) amongst others. There has, however, been no direct demonstration of the intrarenal effects of aldosterone under conditions which exclude the possibility of extra-renal effects. The nearest approach to such conditions is found in the work of Barger, Berlin & Tulenka (1958) and of Ganong, Mulrow & Hollinger (1957). Barger and his collaborators studied the effect of infusions of aldosterone into the left renal artery of the dog. Unilateral effects were observed in some of their experiments, but these were preceded by a long latent period, and no observations were made of the effect of their infusions on the concentrations of electrolytes in plasma. Ganong and his colleagues used larger doses of aldosterone and were unable to show that the kidney which received the intra-arterial injection of hormone responded either more markedly or more rapidly than did the other.

The object of our experiments was therefore to study the renal actions of aldosterone in kidneys perfused with blood and isolated from all nervous and hormonal continuity with the animal body.

METHODS

The cats used were of male, female or neuter sex and weighed from 1·3 to 4·6 kg. Young animals were commonly selected to provide the heart–lung–kidney preparations and the older animals were used as blood donors. Only lactating females or those in advanced stage

of pregnancy were judged unsuitable for use. In all but two experiments the kidney and the heart–lung preparation were taken from the same cat (Lockett, 1959).

Anaesthesia was induced in the heart–lung and blood-donor cats with a mixture of ether and chloroform (1:4 (v/v)) and was maintained in sixteen experiments by the intravenous injection of 8·0 ml. 1% chloralose in 0·9% NaCl per kilogram. In all other experiments anaesthesia was induced with the same mixture of chloroform and ether, but the animals were then all made 'spinal' by passing a blunt rod upwards through the foramen magnum to destroy the medulla, pons, mid-brain and thalamus. This procedure tears the pituitary stalk.

Each experiment began with the collection of heparinized blood (heparin 1500 u./100 ml. blood) for the perfusion circuits from donor cats. Most commonly approximately half of this blood was stored at 7° C during the night and next day until the heart–lung–kidney preparation was made. The first step in this preparation was the cannulation of the left ureter with No. 46 polythene tubing inserted into the renal pelvis through the upper 1–2 cm of the ureter. Next, the kidney was mobilized by ligature and section of any branches of the renal artery or vein which supplied extra-renal tissue and of any small vessels making anastomosis through the renal capsule. Then the aorta and inferior vena cava were prepared for cannulation. All branches except those to and from the left kidney were divided between ligatures from a point 0·5 cm above to 3·0 cm below the left renal branches. Finally, loose ligatures were placed around the abdominal aorta and the inferior vena cava to assist in the very rapid cannulation of these vessels later in the experiment. A heart–lung preparation was then made in the same cat (Lockett, 1957). Immediately after the external circuit of the heart–lung preparation had been established by ligature of the third part of the aortic arch and the inferior vena cava in the thorax, the abdominal aorta and cava were ligated above and cannulated below the origins of the left renal vessels. Then the kidney was swiftly freed by appropriate sections in the ureter, aorta and cava and was perfused through a branch in the external circuit of the heart–lung preparation which arose from a point 5 cm below the peripheral resistance, between it and the brachiocephalic cannula. The renal venous blood was returned by gravity through a 2 mm wide polythene tube to the heart–lung reservoir (Fig. 1). Perfusion of the kidney had begun within 3 min of the ligation of the thoracic aorta which rendered it ischaemic. The preparation was completed in 1–2 hr, according to the time taken for the ureteric cannulation.

There were five types of experiment:

Group I (14 expts.). Anaesthesia was maintained by chloralose, as described above, during the bleeding of the cats and the making of the heart–lung–kidney preparation.

Group II (2 expts.). Anaesthesia as in Group I, but the bleeding of the donor cats was preceded by ligature of the brachiocephalic and left subclavian arteries and was thus effected without there being a circulation through the head.

Group III (5 expts.). After the induction of anaesthesia with chloroform and ether the animals were made spinal as described above after the ligation of the brachiocephalic and left subclavian arteries. Bleeding of the donors and the heart–lung–kidney preparation was thus effected without a circulation through the head.

Group IV (4 expts.). These were prepared as in Group III, except that the brachiocephalic and subclavian arteries were not tied. At the most, the carotid arteries were only temporarily occluded for the moment of spinalization. There was therefore circulation in the head during the bleeding of donors and during the operative preparation.

Group V (4 expts.). The preparations were made under chloralose anaesthesia as in Group I, but the hypophyses were removed from both donor and heart–lung–kidney cats immediately the chloralose had been administered.

Hypophysectomy was begun by the exposure and trephining of the right temporal bone. Then the right side of the vault of the cranium was removed with nibbling forceps, and bleeding from the bone and sinuses was arrested by gentle pressure and bone-wax substitute.

Next the dura was opened to expose the brain and the temporal lobe was lifted so that the optic chiasma and the hypophysis could be seen clearly. The latter was removed with forceps and the resultant bleeding was stopped by gentle pressure before the temporal lobe was lowered into its normal position. Ten minutes later, blood donors were bled from a carotid artery or the operative procedure was begun.

The temperature of the blood and of the kidney chamber varied from 36 to 38° C and the arterial pressure from 126 to 138 mm Hg on different occasions, but these remained constant throughout each experiment. Urine was collected during measured serial periods of time; blood samples were taken at the mid points of these periods from the blood returning to the reservoir through the peripheral resistance (Fig. 1), and were therefore specimens of the

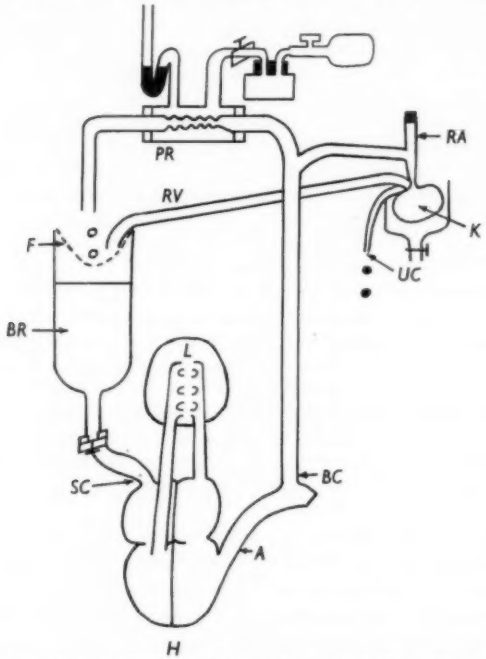

Fig. 1. Diagram of the circuit used to perfuse the kidney from a heart–lung preparation. *A* Aorta, *BC* brachiocephalic cannula, *BR* blood reservoir, *F* filter, *H* heart, *K* kidney, *L* lungs, *PR* peripheral resistance, *RA* renal arterial cannula, *RV* renal vein, *SC* superior caval cannula, *UC* ureteric cannula.

arterial blood supplying the kidney. Hormones were added to the blood in the reservoir 1 min before the end of a period of urine collection. Renal blood flow was measured directly by collecting the venous return from the kidney into a measuring cylinder for a period of time determined by stopwatch.

Creatinine (British Drug Houses, Ltd) was mixed with the blood before the perfusion circuit was filled. Protein-free filtrates of plasma were prepared by the modified tungstate method of Folin & Wu (Smith, 1956) and concentrations of creatinine were estimated both in these filtrates and in urine by the method of Brod & Sirota (1948). The concentration of chloride ions in the urine was determined by the micromethod of Brun (1949) and of sodium and potassium in plasma and urine by means of an EEL flame photometer.

The DL-aldosterone monacetate used was a gift from Ciba Laboratories, Ltd. Synthetic oxytocin (Syntocinon; Sandoz Products, Ltd), natural oxytocin (Pitocin; Parke, Davis, Ltd) and natural vasopressin (Pitressin; Parke, Davis, Ltd) were obtained commercially.

RESULTS

The renal blood flow (RBF) always increased, sometimes fourfold, in the first 20–60 min of an experiment, to reach a plateau level (Fig. 2). The minute volume of urine also increased, becoming constant with or before the RBF in the presence of chloralose (Fig. 2) and with or after the RBF in its absence. This plateau was maintained without great change for several hours (Fig. 2) and during this time the actions of hormones were

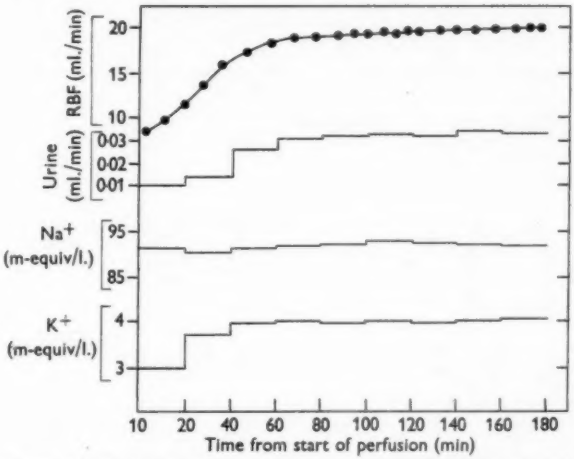

Fig. 2. Renal blood flow, urine volume, and concentrations of sodium and potassium in urine of a kidney perfused without addition of hormones. Kidney weight 7·4 g; an experiment of Group I.

tested. Table 1 shows the values reached in the early stage of the plateau by preparations of the various groups (see above). The effect of aldosterone on renal function depended on the manner of the preparation, but was never accompanied by change in the electrolyte concentrations of plasma.

Actions of aldosterone in experiments of Group I and Group IV

In each case blood was passing through the head during bleeding of the donor animal and until the heart–lung preparation was made; in Group IV the animals were spinal and in Group I under chloralose anaesthesia.

Figure 3 shows the typical result in an experiment of Group IV. Fifty minutes after the experiment began, when the RBF and the rate of urine excretion had reached the plateau, 0·5 μg DL-aldosterone monoacetate was added to the blood in the reservoir to mix with 150 ml. of circulating blood.

After a latent period, which varied from 3 to 5 min in different experiments, the RBF increased to reach a maximum in 10–15 min. Reduction in the minute volume and in the concentrations of sodium and chloride in the urine became apparent in the first 15 min after the addition of aldosterone and reached a maximum in the subsequent period of urine collection. The concentration of potassium in the urine either fell slightly, as in

TABLE 1. Comparison of the function of kidneys perfused with blood from chloralosed, spinal and hypophysectomized (chloralosed) cats, in the absence of added hormone: mean ±S.E. of mean, no. of expts. in brackets

Type	Kidney weight (g)	Renal blood flow (ml./min)	G.F.R. (ml./min)	Urinary excretion (μequiv/min) Na^+	K^+	Cl^-	Urine v (ml./mi
Chloralosed							
Group I: with head circulation	9·6±0·43 (12)	22·2±2·64 (12)	1·61±0·21 (9)	13·3±3·11 (10)	2·4±0·48 (10)	15·2±3·48 (10)	0·06±0 (12)
Group V: hypophysectomized	9·2±0·65 (4)	20·0±2·53 (4)	2·25±0·47 (4)	31·5±5·79 (4)	3·9±0·69 (4)	38·0±7·09 (4)	0·22±0 (4)
Spinal							
Group III: no head circulation	9·9±0·64 (5)	22·6±3·09 (5)	1·76±0·04 (3)	11·2±4·36 (5)	2·0±0·32 (5)	16·8±3·41 (5)	0·05±0 (5)
Group IV: with head circulation	8·5±0·53 (4)	23·0±2·08 (4)	1·70±0·05 (3)	13·5±1·37 (4)	2·3±0·63 (4)	17·0±1·73 (4)	0·03±0 (4)

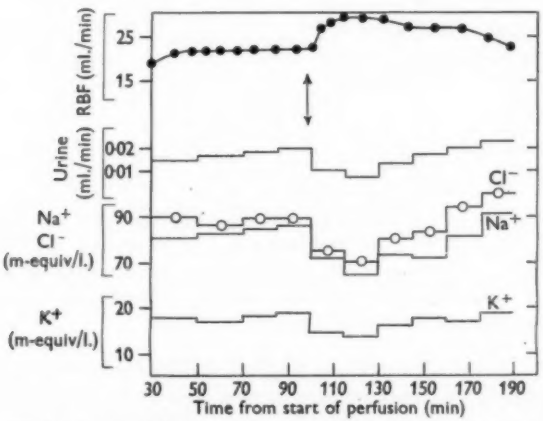

Fig. 3. Effect in an experiment of Group III of aldosterone 0·5 μg at arrow.

this experiment, or remained unchanged; there was always a reduction in the output of potassium ions per minute. In Fig. 3 there was recovery in the composition of the urine in parallel with that in the RBF. More often, however, changes in the RBF have been outlasted by those in the composition of the urine.

The chloralosed preparations were relatively insensitive to aldosterone, so that the doses used in experiments of Group I were five to ten times those in Group IV. The changes induced in renal function were, however, similar in the two groups of experiments.

Figure 4 presents results from an experiment of Group I which shows that a second dose of aldosterone may produce a recognizable effect when the effects of the first dose have not disappeared. Usually about 5–10 μg aldosterone produced maximal changes. There were in this experiment rather transient increases in glomerular filtration rate after each addition of aldosterone; this was a common but not a constant finding. Some

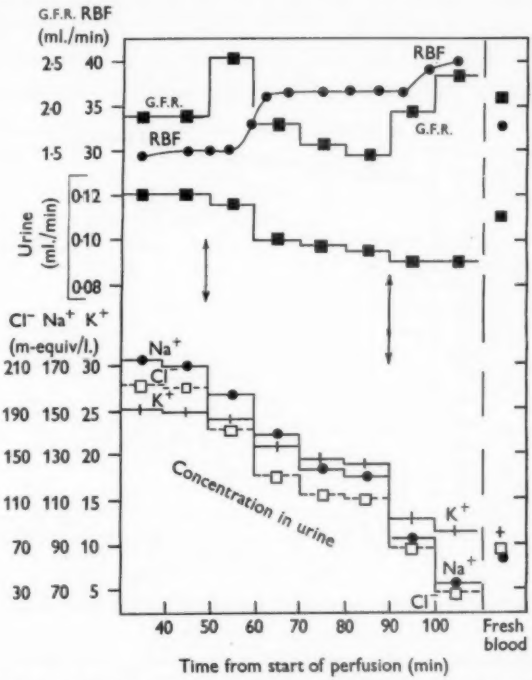

Fig. 4. Effect in an experiment of Group IV of aldosterone 2·5 μg at first arrow, 5 μg at second. From 110 min the kidney was perfused with blood to which no hormone had been added.

recovery in RBF, the minute volume and the concentrations of sodium and chloride of urine was observed 10 min after circulation of fresh blood to which no hormone had been added.

Effects of aldosterone in experiments of Groups II and III

These were experiments in which cats were used with the head circulation excluded during bleeding or preparation. The cats of Group II were under chloralose anaesthesia, those of Group III were spinal. Figure 5 shows the effect of aldosterone in a Group III preparation. There was an increase in RBF in this and in all other experiments. Transient increases in

creatinine clearance were seen in three experiments out of five. The minute volume of urine always increased. Although there was decrease in the concentrations of sodium and chloride in the urine after aldosterone, the diuresis was usually enough to increase the amount of these ions lost per minute. The concentration of potassium in the urine often increased, as in Fig. 5, but sometimes remained unchanged. This diuretic response to aldosterone accompanied by increase in the rate of potassium excretion was characteristic of all experiments of Groups II and III, in which the circulation through the head was excluded during collection of the blood and the operative preparation. It was not solely a response to an initial

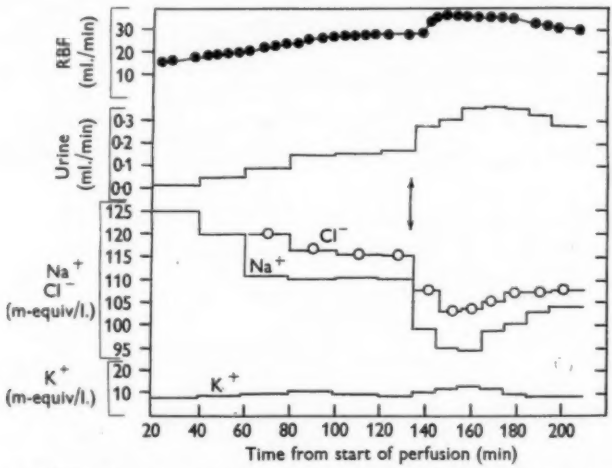

Fig. 5. Effect in an experiment of Group III of aldosterone 5 μg at arrow.

dose of aldosterone, nor to a critical concentration of aldosterone, for it remained apparent throughout the 5 hr of an experiment in which the concentration of circulating aldosterone was steadily raised by the addition of 2·5–5 μg aldosterone monacetate to 160 ml. of circulating blood at intervals of 20–40 min.

The response typical of experiments in Groups II and III (Fig. 5) could be converted to that of experiments belonging to Groups I and IV (Figs. 3 and 4) by oxytocin, as is shown in Fig. 6. The first response to DL-aldosterone monoacetate 2 μg/150 ml. blood produced an increase in the minute volume of urine and in potassium excretion. However, after the addition of 4 m-u. Syntocinon to the circulating 150 ml. of blood, 2 μg aldosterone monacetate caused reduction in the minute volume of urine and in the amount of potassium excreted per minute. In other words, the effect of aldosterone on the kidney perfused with blood that had not recently circu-

lated through the head was converted, by the addition of oxytocin, to an action of aldosterone typical of this hormone on kidneys perfused with blood which had recently circulated through the head. The naturally occurring oxytocin, Pitocin, was as effective as the synthetic preparation in this respect.

Effects of aldosterone in experiments of Group V

Four experiments were made in which the kidney from a cat hypophysectomized under chloralose anaesthesia was perfused with blood

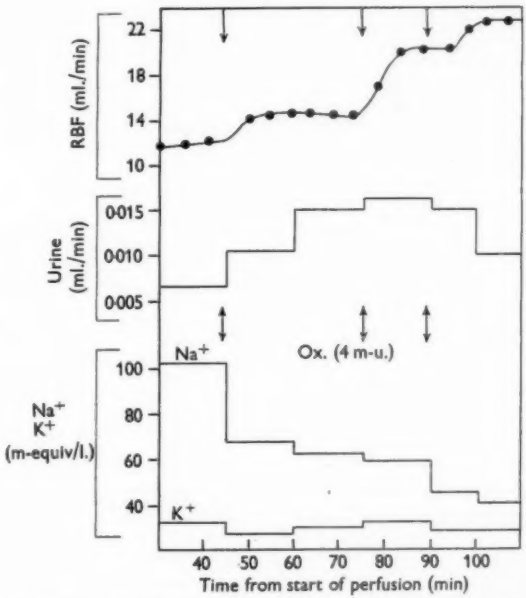

Fig. 6. Effect in an experiment of Group III of aldosterone, 2 μg at first and third arrows, and its modification by oxytocin 4 m-u. at second arrow.

collected from other cats which had been hypophysectomized under chloralose shortly before being bled. In every case the addition of 2–7 μg aldosterone monoacetate to 150 ml. of circuit blood caused an increase in RBF, a diuresis and decrease in the concentrations of sodium and chloride with increase or little change in that of potassium in the urine (Fig. 7). The response was like that in preparations of Groups II and III, which were made without a circulation through the head (Fig. 5). Moreover, synthetic oxytocin 10 m-u./150 ml. blood converted this diuretic action of aldosterone to one of antidiuresis accompanied by potassium retention, characteristic of the action of this hormone in preparations made in the

presence of a circulation through the head (Groups I and IV, Figs. 3 and 4). This conversion could also be effected by 50 m-u. of vasopressin, as was well shown by the experiment in Fig. 8, in which the circuit blood was divided into two portions. One part was used for the study of the effect of vasopressin on the responses of the preparation of aldosterone. The blood used for this part of the experiment was then replaced by the second portion of the same blood, and later, when the kidney was fully acclimatized to the change, examination was made of the effect of oxytocin. After 5 m-u. of oxytocin, aldosterone produced antidiuretic effect.

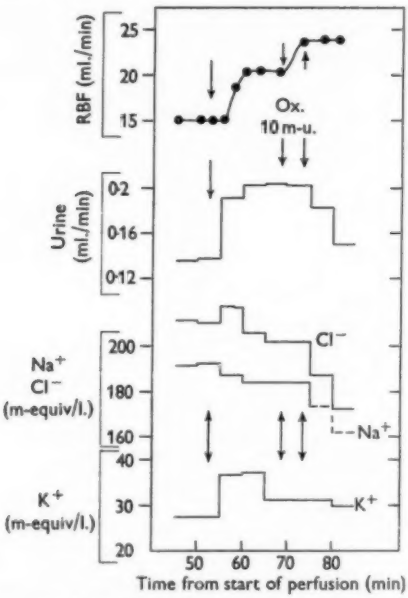

Fig. 7. Effect in an experiment of Group V of aldosterone 7 μg at first and third arrows, and its modification by oxytocin 10 m-u. at second arrow.

Actions of oxytocin and vasopressin

The actions of oxytocin and vasopressin alone have also been investigated. It should be noted that the heart–lung–kidney preparations in which the effects of the neurohypophysial hormones were studied had already been used for investigation of the actions of aldosterone, and that the long-lasting changes induced in the concentrations of urinary electrolytes by the adrenal hormone had only rarely been fully reversed when the pituitary hormones were administered.

Oxytocin. Since oxytocin has been shown to be released during haemorrhage in rats (Ginsburg & Brown, 1957) the direct actions of oxytocin on

the kidney were examined only in preparations of Groups II, III and V, in which the initial concentrations of oxytocin were probably low. Oxytocin (4–25 m-u.) added to 150 ml. of such blood increased the RBF and the clearance of creatinine but failed to influence either the concentrations of electrolytes or the minute volume of urine (Table 2 and Fig. 9). There

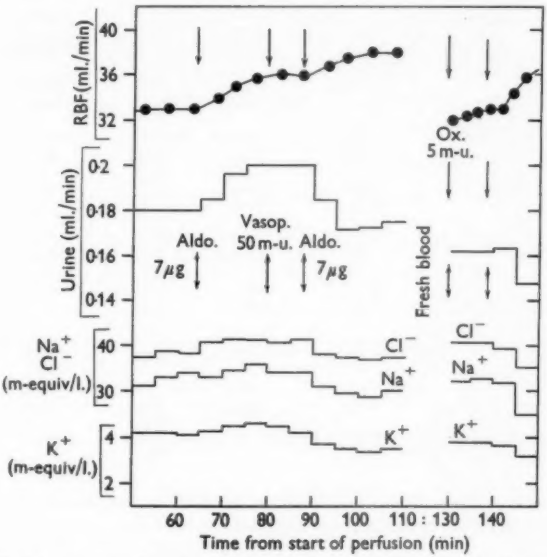

Fig. 8. Effect in an experiment of Group V of aldosterone 7 μg at first, third and fifth arrows, and its modification by vasopressin 50 m-u. at second arrow. The kidney was perfused with fresh blood at 120 min. At fourth arrow, oxytocin 5 m-u.

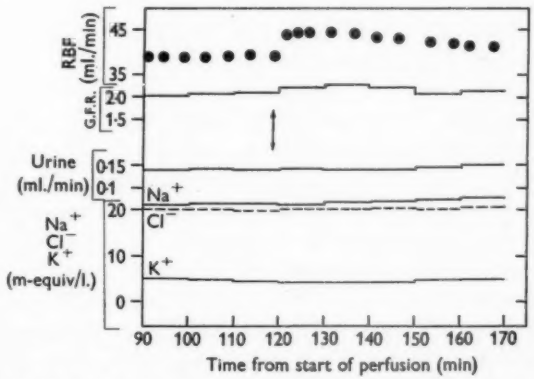

Fig. 9. Effect in an experiment of Group V of oxytocin 10 m-u. at arrow. Plotting as in Fig. 2.

was no apparent difference in the effect of these concentrations of oxytocin in the presence and absence of chloralose anaesthesia.

Vasopressin. In preparations of Group III (spinal, without a head circulation) small doses of vasopressin produced decrease in urine flow,

TABLE 2. Effect of oxytocin on the isolated perfused cat kidney

Type of preparation	Oxytocin (m-u./150 ml.)	Urine (min vol)	RBF (ml./min)	G.F.R. (ml./min)	Na^+ (μequiv/min)	K^+ (μequiv/min)
Group III	4	0·016	14·4	1·63	93·0	5·1
		0·017	19·0	1·69	98·0	3·4*
Group III	20	0·056	28·0	2·20	57·0	3·0
		0·060	33·5	2·59	65·0	3·3*
Group II	4	0·36	46·0	2·41	37·5	6·5
		0·36	48·0	3·00	37·5	6·9*
Group V	4	0·66	35·2	3·14	87·5	6·8
		0·76	42·4	3·62	89·2	6·9*
Group V	5	0·13	32·8	1·40	20·7	3·7
		0·14	36·6	1·70	21·8	3·7*
Group V	10	0·22	20·4	3·17	40·2	7·3
		0·22	24·0	3·49	40·8	7·3*
Group II	25	0·17	26·4	1·60	20·4	4·6
		0·18	30·6	1·93	22·5	4·6*

* In an observation period of 20–30 min after oxytocin had been added to the circulating blood.

TABLE 3. The effect of chloralose anaesthesia on the sensitivity of the kidney to antidiuretic hormone

Type of preparation	Vasopressin for threshold effect (m-u./150 ml.)	Vol. urine (ml./min)	RBF (ml./min)	Urinary electrolytes (μequiv/min) Na^+	K^+	Cl^-
Group II	4	0·032	16·2	50	2·0	56
		0·037	16·2	50	2·0	58*
Group II	20	0·014	28·0	14	2·0	15
		0·016	30·0	16	2·3	18*
Group V	8	0·050	22·6	60	1·4	73
		0·035	22·6	50	1·0	65*
Group V	10	0·040	21·8	12	1·1	—
		0·060	21·8	23	1·2	—*
Group V	10	0·025	24·0	24	1·2	27
		0·040	23·5	26	1·4	31*
	(μu./150 ml.)					
Group III	20	0·36	24·4	106	9·5	—
		0·20	24·4	95	4·6	—*
Group III	20	0·31	20·0	37	3·0	38
		0·28	21·0	35	3·0	36*
Group III	25	0·24	22·6	62	2·8	65
		0·18	23·0	60	3·0	62*

* After vasopressin had been added to the circulating blood.

20–25 μu./150 ml. blood evoking threshold effect (Table 3). In the presence of chloralose anaesthesia in preparations of Groups V and II much larger doses failed to produce antidiuresis (Table 3). Chloralose anaesthesia therefore very greatly reduced the sensitivity of the kidney to vasopressin.

DISCUSSION

The classical effect of aldosterone in the intact animal is one of sodium retention and potassium loss (Mach, Fabre, Duckert, Borth & Decommun, 1954). It has not been possible to demonstrate this on the isolated kidney in any of the selected groups of experimental circumstances. In experiments of Groups I and IV, in which the blood was drawn and the preparation made whilst blood circulated through the head, aldosterone caused

Table 4. Changes induced by aldosterone in the excretion of sodium and potassium by the isolated perfused cat kidney: mean ±S.E. of mean, no. of expts. in brackets

Type of preparation	Aldosterone (μg/150 ml. blood)	Percentage change in urinary electrolytes: Amount excreted per minute Na	Amount excreted per minute K	Concentration per litre Na	Concentration per litre K
Group I	10	−53±3·5 (4)	−34±5·2 (4)	−18±3·9 (4)	−11±3·3 (4)
	5	−37±3·0 (4)	−37±4·8 (4)	−23±6·5 (4)	−15±3·2 (4)
Group III	2	36±7·1 (4)	52±9·8 (4)	−21±7·9 (4)	17±4·7 (4)
Group IV	2	−30±9·0 (3)	−44±3·5 (3)	−20±1·1 (3)	−22±6·8 (3)
Group V	7	3±1·1 (4)	39±5·3 (4)	−9±1·2 (3)	21±3·7 (3)

Only data derived from renal responses to the first addition of hormone to the perfusing blood have been used in the compilation of this table.

antidiuresis and reduction in the rate of excretion of both sodium and potassium. When the head was excluded from the circulation (Group III) or the hypophysis was removed (Group V) before bleeding and preparation, aldosterone caused diuresis and increased the rate of excretion of both potassium and sodium (Table 4). The difference in the action of aldosterone in Groups I and IV on the one hand and Groups II, III, and V on the other is most probably to be attributed to differences in the concentrations of an oxytocin-like substance present, since the response given by preparations of Groups III and V was regularly converted to that characteristic of Groups I and IV by the addition of oxytocin to the circulating blood. The nearest approach to the action of aldosterone in the whole animal was seen in preparations of Groups III and V. In these the hormone caused a fall in the sodium and a rise in the potassium concentrations of the urine (Table 4) but this was accompanied by diuresis. No such diuresis accompanies the action of physiological doses of aldosterone in the intact cat (unpublished observations).

Chloralose failed to alter the sensitivity of the isolated kidney to

oxytocin (Table 2), caused some decrease in sensitivity to aldosterone without effecting qualitative change in the actions of this hormone (Table 4) and grossly decreased renal sensitivity to vasopressin (Table 3). It may therefore be concluded that chloralose, in the concentrations used, had little effect on the renal blood vessels, only slightly depressed the active reabsorption of sodium and potassium excretion, but profoundly inhibited the facultative reabsorption of water in the distal nephron by preventing those changes in cellular permeability to water brought about by vasopressin (Wirz, 1953).

SUMMARY

1. The actions of DL-aldosterone monoacetate 0·5–10 μg/150 ml. of circulating blood have been examined in cats' kidneys perfused from heart–lung preparations.

2. If either in spinal cats or under chloralose anaesthesia blood was circulating through the head during the operative preparation of the heart–lung–kidney preparation and during collection of the blood for the circuit, aldosterone caused an increase in renal blood flow, decrease in the urine volume, and decreases in the urinary concentrations of Na, K, Cl.

3. If either in spinal cats or under chloralose anaesthesia the circulation through the head was excluded during the bleeding and operative preparation, aldosterone caused an increase in renal blood flow, an increase in the urine volume, little change in K and decreases in Na and Cl concentrations in the urine. In consequence there was an increase in Na and Cl excretion, and a smaller increase in K excretion. Aldosterone produced a similar effect when hypophysectomized animals were used.

4. The effect of aldosterone was changed from that in (3) to that in (2) above by oxytocin 10 m-u./150 ml. blood. Vasopressin was less effective.

5. Oxytocin (1–20 m-u./150 ml. blood) did not alter the volume or electrolyte composition of the urine.

6. Chloralose reduced the sensitivity of the kidneys to vasopressin. In the absence of chloralose vasopressin (15–20 μu./150 ml.) reduced urine flow; in the presence of chloralose, 4–20 m-u. had no effect. There was no effect on electrolyte excretion.

7. Chloralose made the kidneys only slightly less sensitive to aldosterone and did not influence the character of the response.

This work was undertaken whilst one of us, M.J.D., was receiving a grant for personal maintenance from the Pharmaceutical Society of Great Britain for Training in Research.

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INNERVATION AND ACTIVITY OF A MOLLUSCAN SMOOTH MUSCLE

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The anterior byssal retractor muscle of *Mytilus edulis*, L. is capable of two types of contraction, a brief contraction which persists only during stimulation and a prolonged contraction which continues long after stimulation has ceased. The mechanism by which the prolonged contraction is maintained is in question. It has been suggested that a 'catch' mechanism operates, as originally suggested by von Uexküll (1912), wherein a prolonged state of resistance to stretch is instituted, which does not depend on continuing re-excitation as in a tetanus. Supporting a catch mechanism are observations of continued contraction in the absence of the electrical potentials and membrane depolarization which accompanied tension development (Fletcher, 1937*b*; Twarog, 1954). It has also been suggested that contraction is maintained by repeated re-excitation at intervals appropriate to the slow rate of relaxation of single twitches in this muscle, i.e. that it is a tetanus (Hoyle & Lowy, 1956; Abbott & Lowy, 1958). With high amplification Hoyle and Lowy observed electrical potentials during sustained contraction. These potentials seemed to originate locally at many points along the muscle. Increase in their frequency appeared to be correlated with tension maintenance. Relaxation generally coincided with decreased small potential frequency. It was concluded that the small potentials represented highly localized excitatory activity, possibly myogenic but probably initiated via an intramuscular ganglionic plexus (Hoyle, 1957). Relaxation was attributed to inhibition of the excitatory activity. In explanation of instances where electrical activity was not reduced during relaxation, Hoyle & Lowy (1956) suggested that, because of the localized nature of the potentials, activity at the electrodes represented an insignificant number of active units in an otherwise quiescent muscle. It was also considered that relaxation could involve simple inhibition, as in crustacea (Marmont & Wiersma, 1938).

In the present study electrical activity was recorded simultaneously at many points along the length of the muscle during rest, tension development, tension maintenance and relaxation. The gross innervation of the

byssus retractor muscle was traced morphologically and physiologically. A nerve–muscle preparation was developed and electrical and mechanical responses to neural stimulation observed. The origin and distribution of both high- and low-voltage electrical potentials was studied.

METHODS

Anterior byssal retractor muscles of large specimens of *Mytilus edulis* L. were dissected and prepared for recording tension and electrical activity in various ways as explained individually in the Results section. For recording tension the muscle was attached to a strain gauge. A 5–10 g weight kept the muscle under constant stretch during the experiment. While contraction was not strictly isometric, shortening was limited to approximately 2 mm by the transducer. Recording electrodes consisted of chlorided silver wire mounted in polyethylene tubes filled with sea water. Cotton wicks made contact with the muscle. For stimulation the cut nerves were pulled by suction into a finely tapered polyethylene tube filled with sea water, in contact with a chlorided silver wire. A second chlorided silver electrode passed around the nerve just outside the orifice of the tube. The nerves which were stimulated are very small and lie close to the muscle; with the suction electrode excellent localization of current flow was obtained.

For two-channel recording, a Grass Model 5-Polygraph was used. The output of an FT-02 force-displacement transducer was led to a Grass 5-P1 low-level DC pre-amplifier and finally displayed on one channel of the ink-writing oscillograph. Recording electrodes were led directly to a 5-P1 low-level DC pre-amplifier and electrical activity displayed on the second channel of the ink-writer. Maximum sensitivity was 10 μV/cm deflexion; frequency response was linear between 0 and 45 c/s, falling to 1/2 at 60 c/s. The pre-amplifier was also used capacitor-coupled, with a time constant of 0·8 or 0·1 sec.

For multichannel recording a Grass Model III eight-channel electroencephalograph was used. The output of the transducer was in this case led to a Grass Model 2 balance demodulator installed in the eighth channel. The electrodes were led to the e.e.g. through alternate-coupled amplifiers. Maximum sensitivity was 10 μV/cm deflexion, the frequency response was linear from 0·5 to 75 c/s, falling to 1/2 at 100 c/s.

The rapid asynchronous potentials observed by Hoyle & Lowy (1956) were 20–60 msec in duration, well within the range of linear frequency response of both electroencephalograph and polygraph. While single nerve action potentials could not be recorded adequately by these instruments, the most rapid spike potentials known in smooth muscle cells (8–15 msec in intracellular recordings by Holman, 1958) would have been detected by the electroencephalograph.

RESULTS

Nerve supply: morphological and physiological tracing

Although the brightly coloured (yellow-orange) pedal and cerebral ganglia are easily seen in the living *Mytilus*, nerves are very slender and are nearly of the same colour as the muscle. Selective staining with leucomethylene blue (Pantin, 1948) proved successful. Even more useful was the simple method of exposing the ganglia and nerve trunks, then adding 10% HNO_3 in sea water as the dissection progressed. Although the tissue became more difficult to dissect cleanly, the gleaming white colour of the nerves freshly treated with HNO_3 greatly simplified tracing them. Results of this study are shown in Fig. 1. It was possible to follow the

two branches of the visceral nerve into the dorsal and ventral bundles, respectively, of the anterior byssus retractor muscle. The major nerves leading from the cerebral and pedal ganglia were traced and did not seem to penetrate the muscle at any point. These observations are at variance with studies on European *Mytilus edulis*, L. by Van Nieuwenhoven (1947), who found that the ventral byssus retractor nerves sent branches into the anterior retractor muscles at the byssal end, and by Hoyle & Lowy (1956) who stated that the muscle is innervated 3 mm from the byssal end. However, the innervation described in the present study coincides with the description by List (1902) of the innervation of the anterior byssal retractors of *Mytilus galloprovincialis*, a closely related species.

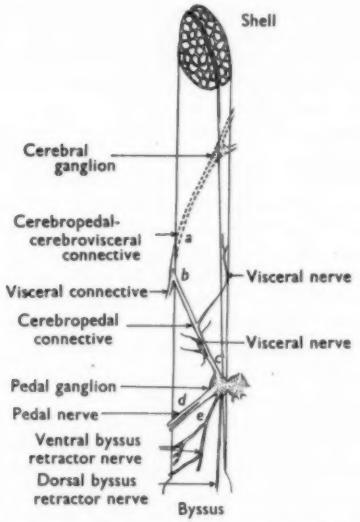

Fig. 1. The anterior byssal retractor muscle of *Mytilus edulis* L., dorsal aspect. Associated ganglia and gross innervation.

Lack of agreement as to the innervation of the muscle made a physiological study desirable. The problem was first approached by suspending the muscle (cleaned from ganglia and large nerve trunks) in a moist chamber, and recording tension. Closely spaced (4 mm) silver–silver-chloride electrodes were moved over the muscle surface. Stimuli of suprathreshold voltages, 20 msec in duration, were applied for 5 sec at a frequency of 5/sec, at various distances from the end of the muscle. With electrodes within a centimetre of each end of the muscle tension development was minimal, while with electrodes in the mid region the response rose sharply to a maximum. The region of maximum response involved

an area of about $\frac{1}{4}$ to $\frac{1}{3}$ of the total muscle length. A similar experiment was performed with 2 mm squares of Whatman No. 1 filter paper soaked in 10^{-4}M acetylcholine, which were placed on the muscle for 2 min. Tension development was minimal when the acetylcholine was applied at either end of the muscle. The maximal response in the mid region was not so sharply delimited as with electrical stimulation. Possibly diffusion prevented true localization of the stimulus. A series of muscles were compared before and after eserinization and soaking in acetylcholine. The muscles were suspended in air and the potential difference measured between the byssal end and points along the muscle. The maximum potential difference recorded was 2 mV, a 1 mV difference being more common. In 8 out of 12 experiments a region of maximum depolarization occupied the central third of the muscle. In the other experiments the depolarization was more evenly distributed.

These experiments indicated that low thresholds for both electrical and chemical excitation exist in the mid region of this long muscle. The results of electrical stimulation differ from observations of Hoyle & Lowy (1956) who found consistently low thresholds at the byssal end.

Finally, development of the small suction electrode which is described in the Methods section permitted nerve tracing by localized electrical stimulation. All nerves associated with the cerebral and pedal ganglia could be exposed, individually stimulated, then divided and the stumps both proximal and distal to the ganglia again individually stimulated. The animal was opened by cutting the adductor muscles and held open by a wire device. One shell was held in a femur clamp and the anterior byssal retractor muscle on that side was isolated at the byssal end from the other byssal muscles (all cuts were made well posterior and lateral to the insertion of the muscle). The free byssal end was connected to the right-angle lever for tension recording. Results are summarized in Table 1.

These results suggest that the excitatory innervation of the anterior byssal retractors comes entirely from the cerebropedal connective between the pedal ganglion and the branching of the visceral connective, probably through the visceral nerves. Apparently excitor fibres from the cerebral ganglion and the pedal and ventral byssus retractor nerves pass through the pedal ganglion to the visceral nerves or make synapses in the pedal ganglion with neurones which enter through the visceral nerves.

Responses to neural stimulation; a nerve–muscle preparation

On the basis of the nerve tracing, a nerve–muscle preparation was developed. The cerebral ganglion was removed and the cerebropedal-cerebrovisceral connective divided near *a* (Fig. 1). All nerves to the pedal ganglion except the cerebropedal connective were cut. The pedal

ganglion was lifted and the attached cerebropedal connective was freed at its posterior end from surrounding connective tissue. Adhering tissues were removed, except in the region of the innervation since it was very difficult to avoid damage when exposing the visceral nerves. The muscle was suspended for recording. The cerebropedal connective was then divided at its juncture with the pedal ganglion and the large resulting contraction observed. If there was no contraction, the preparation was assumed to be injured and was discarded. Histological studies (List,

TABLE 1

Stimulate:	Pedal and cerebral ganglion intact	Cerebral ganglion removed	Pedal ganglion removed	Visceral nerves divided*
Cerebral ganglion	(+)		0	–
Cerebropedal—cerebro-visceral connective	(+)	(+)	0	–
Visceral connective	0	0	0	–
Cerebropedal connective (Divided at *b*)				
Proximal segment	(+)	–	–	0
Distal segment	0	–	–	0
(Divided at *c*)				
Proximal segment	0	–	–	0
Distal segment	++		++	0
Pedal ganglion	++	++	–	0
Pedal nerve (divided at *d*)				
Proximal segment	++	–	0	–
Distal segment	0	–	–	–
Ventral byssal retractor nerve (divided at *e*)				
Proximal segment	++	–	0	–
Distal segment	0	–	–	–
Dorsal byssal retractor nerve	0	–	–	–

* Visceral nerves were divided in only three experiments as these were very difficult to see. (+) Weak, unreliable contraction; ++ strong contraction; 0 no response; – not observed.

1902; H. W. Deane & B. M. Twarog, unpublished) have shown that this preparation inadvertently included nerve cell bodies in the nerve trunk, especially at the branching of the cerebrovisceral and cerebropedal connectives (near *b* of Fig. 1). The number of cell bodies could have been reduced but unfortunately this was realized too late. The free end of the cerebropedal connective was pulled into the stimulating electrode, and the wick electrodes arranged as shown in the inset of Fig. 2.

All electrical stimuli, whether brief or prolonged, repetitive or single, led to a contraction which relaxed promptly. Only excitation was ob-

served, although attempts were made to separate relaxing effects from fast and possibly slow contraction by systematically altering the voltage, duration and frequency of stimulation. With the pedal ganglion intact weak stimuli often resulted in relaxation, as stated by Van Nieuwenhoven (1947) and Hoyle & Lowy (1956) but the effect was not reliable in our preparation.

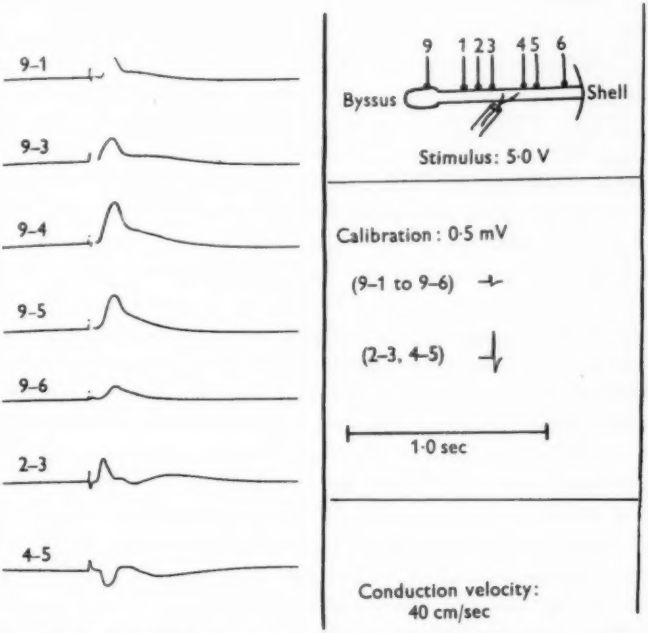

Fig. 2. Electrical responses to stimulation via the visceral nerves. Inset, stimulating electrode on cerebropedal connective; recording electrodes spaced along muscle.

In the experiment of Fig. 2, single stimuli were given (5 V, 1 msec duration, suction electrode positive) and the electrical response was recorded simultaneously at points along the muscle length. Potentials at electrodes 1, 3, 4, 5 and 6 were measured with reference to electrode 9 at the byssal end, and were essentially monophasic, while between electrodes 2 and 3 and 4 and 5 the potentials were diphasically recorded. The diphasic records indicate that the impulse travelled from the central region to either end, electrode 3 becoming negative to 2 and electrode 4 negative to 6 (see also Figs. 3 and 4). In the monophasic records the electrical potential decreased in amplitude between the mid region and either end of the muscle. The largest, most prolonged negativity appeared at the region of

nerve entry, at electrode 4. Apparent conduction velocity averaged 40 cm/sec at 24° C.

The origin and distribution of small potentials; correlation with tension development and maintenance

As seen above, development of tension in response to electrical and to chemical stimulation was invariably preceded by depolarization, of the order of 0·5–5 mV. During rest and maintained tension no depolarizations of this magnitude were recorded. Potential changes of a lesser magnitude would not be detectable at the amplification used, as pointed out by Hoyle & Lowy (1956). Therefore observations were made at increased amplification, using the nerve-muscle preparation exactly as described above, except that recording was begun with the pedal ganglion intact and continued during and after section of the cerebropedal connective (and total removal of the pedal ganglion). Recording continued while the preparation was stimulated through the nerve as described above and finally after all nervous and connective tissue had been cleaned off. In a few experiments the muscle was then teased down to a small bundle. As may be seen in the accompanying figures, multichannel records of responses to nerve section and neural stimulation were made at low gain, to keep on scale, while activity during rest and after nerve section was recorded at high gain. High gain was used throughout in the two-channel studies, and initial responses were permitted to go off scale.

Typical results are seen in Fig. 3. Small spike-like electric potentials were abundant while the pedal ganglion was intact. These potentials were very prominent in the first minutes after removing the pedal ganglion (Fig. 5*a*, *b*; Fig. 7*b*) but then decreased to a very low level of activity (Fig. 3). In the stripped muscle these potentials were detected at low frequency. They were very rare or absent in the teased bundles. As noted above, the nerve trunks included in this preparation were not free from ganglion cells even after removal of the pedal ganglion. The teased muscle bundles were probably free from nerve trunks. Especially to be noted in Fig. 3 is the complete absence of localized activity. Although comparison of the diphasic channels (2–3 and 5–6), ignoring the monophasic channels, might suggest localization, the polarity of the responses which are common to these diphasic channels suggests propagation from intervening areas. When the monophasic channels are examined, it becomes apparent that *without exception, activity appearing at the diphasic electrodes is represented at the centrally placed monophasic electrodes.* The absence of localized activity is perhaps more convincing in Fig. 4, where diphasic recordings were made along the length of the muscle. When the shape and distribution of these small potentials are compared with the large spike-

like potentials produced by neural stimulation, a detailed similarity is evident. In Fig. 3 the size of potentials in the monophasic records coupled with the size and polarity of potentials in the diphasic records suggests

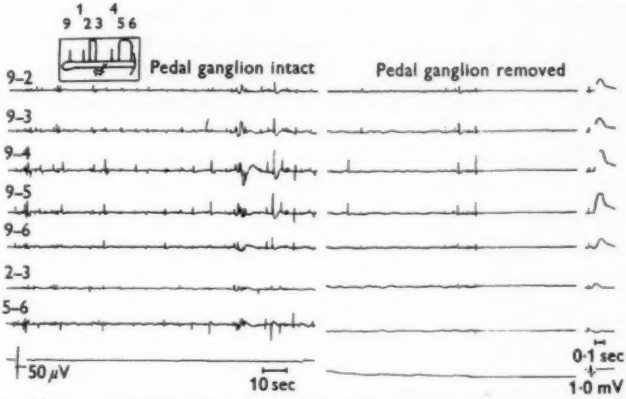

Fig. 3. Spontaneous electrical activity before and after removal of pedal ganglion. Upper five channels, monophasic; two diphasic channels below; bottom channel, tension. Response to stimulation of cerebropedal connective shown at right. Inset, distribution of recording electrodes.

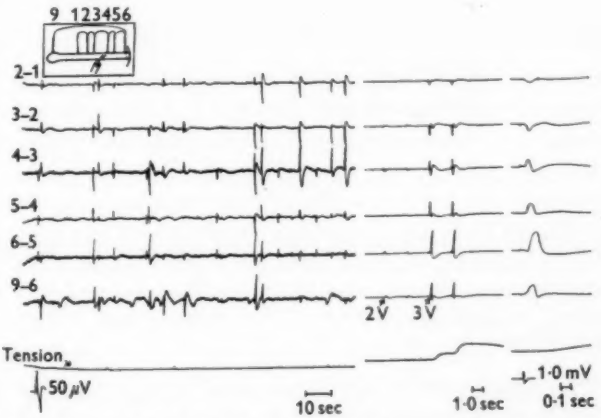

Fig. 4. Spontaneous electrical activity in a resting muscle, recorded diphasically. Neurally evoked responses, recorded at the same electrodes, at centre and on the right. Bottom tracing, tension. Inset, distribution of electrode pairs along muscle.

that the electrical activity originated near electrode 4 and was conducted to either end of the muscle; while in Fig. 4 the polarity reversal between electrodes 3 and 4 and either end of the muscle indicates that negativity

arose between 3 and 4, probably nearer 3, and was conducted to either end.

Briefly, the presence of low-voltage electrical activity, as described by Hoyle & Lowy (1956), is confirmed. However, the potentials recorded in the present study are not localized. As with the large neurally-evoked potentials, no potentials appear distally which are not initially represented at centrally placed electrodes. The records show that they arise first at the point of entry of the nerve supply and diminish in amplitude

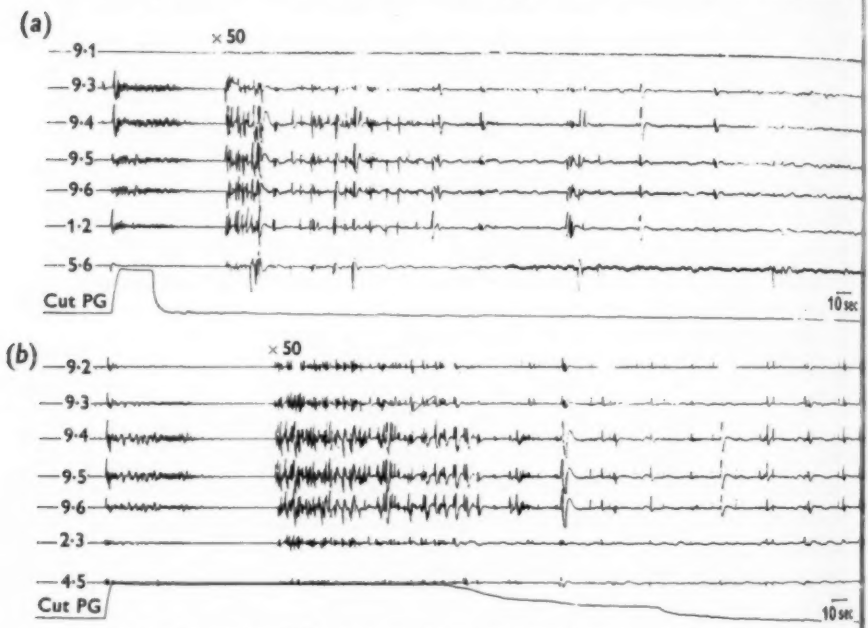

Fig. 5. Electrical activity during and immediately after removal of pedal ganglion (PG). Upper five channels, monophasic records, diphasic records below; bottom channel, tension. Amplification increased fiftyfold following nerve section. (*a*) Brief contraction. (*b*) Prolonged contraction. Note similar course of diminution in frequency of 'large' and 'small' potentials.

distally. The frequency of their occurrence in the 'resting' muscle is greatest in the presence of the pedal ganglion or after nerve injury.

Correlation of the frequency and amplitude of these small potentials with tension maintenance was difficult. With respect to the contraction evoked by section of the cerebropedal connective (Fig. 5*a*, *b*) tension development was seen to be associated with a gradually diminishing burst of large spike-like potentials. When these potentials became infrequent, a fiftyfold increase in amplification revealed small potentials at

a frequency much above the resting level. A close relationship between tension and frequency was often seen (Fig. 5*b*) and comparison of Fig. 5*a* and *b* shows that the absolute activity level was less in the muscle which relaxed rapidly. However, examination of a variety of similarly made records, of which these are selected as extreme examples, makes it difficult to feel certain that the maintained tension level is in any absolute way correlated with small potential frequency. In fact, the muscle in Fig. 5*a* relaxed before large-potential activity had ceased. Muscles which contract weakly show a similar pattern of discharge. This is quite certainly an injury discharge, and it is troubling to consider that in most preparations it may only by chance relate to tension maintenance.

Direct examination of this correlation is beset by difficulties. A prolonged contraction, as mentioned above, is not generally obtained by neural stimulation. With the pedal ganglion intact small-potential frequency is fairly high and can be compared before and after electrical stimulation. Long cathodal stimuli often do not produce sustained contractions when the pedal ganglion is present. With the stripped preparation small potentials are very infrequent either at rest or after cathodal d.c. (during prolonged contraction).

With acetylcholine, sustained tension is produced whether the pedal ganglion is present or not. Furthermore, it is possible to record continuously the electrical activity before, during, and after application of acetylcholine by using a system such as that shown diagrammatically in Fig. 6. The validity of this type of recording depends on the evidence that the small potentials are not local, but initiate in the mid region and conduct with varying reduction in amplitude to both ends. Activity would ideally be recorded with one electrode on a crushed end and the other in contact with the site of origin of impulses. With the muscle immersed in a sea-water bath, only impulses reaching the sea-water–air interface could be recorded. This less ideal method was considered adequate for the experiment of Fig. 6 where it had the advantage of avoiding artifacts due to draining the chamber. The muscle was in air throughout the experiment of Fig. 7 to take advantage of the more favourable recording arrangement. Control (sea-water) and test solutions were dropped on to the muscle, thus creating an artifact which obscured the first stages of the response. It should further be noted that distortions were introduced by the use of condenser coupling. The muscle in Fig. 6, with the pedal ganglion intact, had been treated with a high concentration of 5-hydroxytryptamine before applying acetylcholine, in order to emphasize the spiking activity. Tension development (judging from preceding records) began just after the onset of depolarization, before the large spikes. The electrical responses of Fig. 6 were led off in parallel from the same electrodes, through direct-

and alternate-coupled amplifiers, respectively. It is readily apparent that the slow depolarization is no longer seen, the duration of the negative phase of the response is shortened and a diphasic artifact appears as a result of condenser coupling. The advantage of being able to record both small and large spikes without going off scale outweighs, for the special purpose of these experiments, the disadvantages. However, any detailed comparison of the time course or shape of these distorted potentials with those of Fletcher (1937*a*, *b*) is not possible. This applies also to the work of Hoyle & Lowy (1956), who made the comparison although they used condenser coupling very similar to ours.

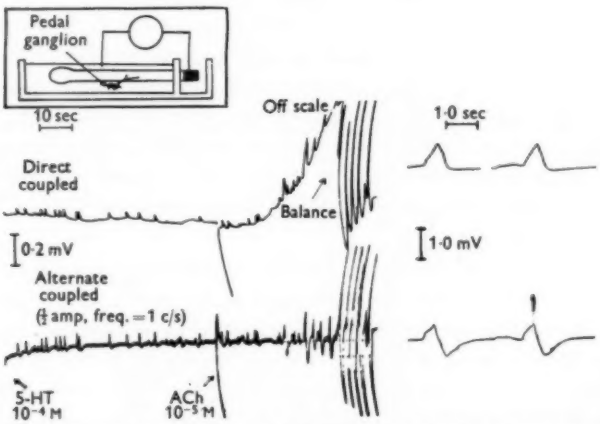

Fig. 6. Electrical activity in 10^{-4} M 5-HT before and during application of ACh. Responses recorded with direct-coupled (upper trace) and capacitor-coupled (lower trace) amplification. Note distortions due to capacitor coupling. Note abundant 'small' potentials (ganglion intact).

Figure 7 shows the effect of acetylcholine and 5-hydroxytryptamine on electrical activity and tension: (*a*) with the pedal ganglion intact, (*b*) during the first few minutes after removal of the pedal ganglion and (*c*) in the stripped muscle. The somewhat greater frequency of small potentials in the presence of acetylcholine was clearly not proportional to tension maintenance in any case. In Fig. 7*a*, the tension developed in the presence of acetylcholine was poorly sustained following 5-hydroxytryptamine, but small-potential frequency was equally high. Tension was least well sustained in the first minutes after nerve section, when small-potential activity was prominent. At this time 'spontaneous' brief contractions were frequent. 'Spontaneous' tension development was always preceded by large, off-scale depolarizations. In the stripped muscle the tension developed was usually doubled for a given dose of acetylcholine (note

change in scale) and was sustained better than in preparations with intact ganglia. Spike-like potentials were more numerous after 5-hydroxytryptamine reliably in the case of the off-scale large deflexions, and often in the

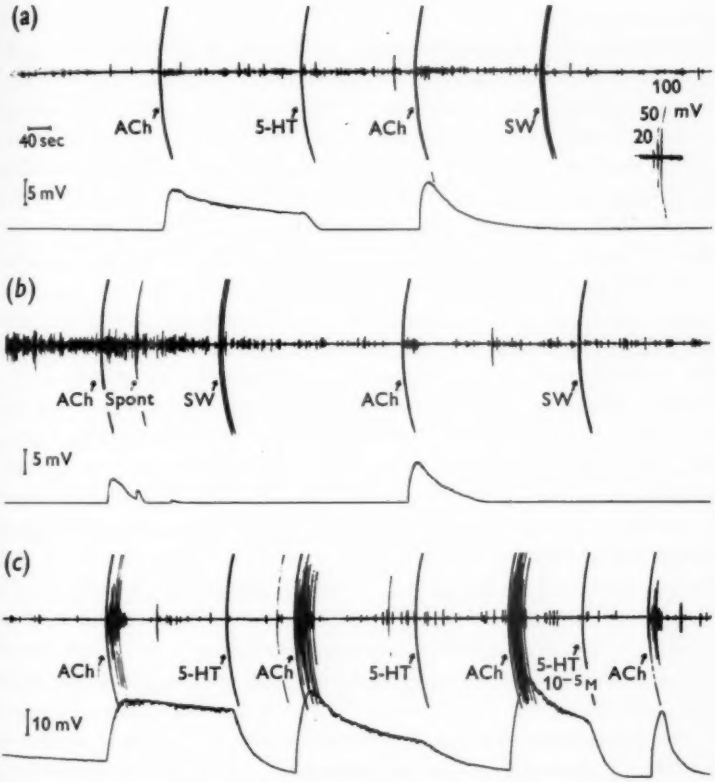

Fig. 7. Electrical activity at mid region of muscle at high amplification, capacitor-coupled. Tension, calibrated in arbitrary units, lower trace. ACh and 5-HT 10^{-7} M applied as indicated: SW, sea water. (*a*) Pedal ganglion intact; note 'small' potential frequencies during contraction, relaxation. (*b*) Immediately following removal of pedal ganglion; note poorly sustained contractions, spontaneous contraction. (*c*) Stripped muscle; note increased tension development (scale changed), well maintained contractions, prominence of 'large' potentials.

case of small potentials. The tension developed was roughly in proportion to the number of off-scale and nearly off-scale deflexions (particularly compare the last contraction of Fig. 7*c* where application of a high concentration of 5-hydroxytryptamine depressed the acetylcholine response). However, tension maintenance did not show any clear proportionality

to either increase or decrease in frequency of small potentials. High concentrations of 5-hydroxytryptamine did not appear to block small potential activity in any case (e.g. Fig. 6).

DISCUSSION

Excitatory nerves appear to enter exclusively in the mid region of the elongate byssal retractor muscle. Electrical activity recorded in this study, both small and large potentials, started in the mid region and appeared to conduct, with reduction in amplitude, to the ends of the muscle. Small-potential frequency was high immediately following nerve injury and very low after ganglia and major nerve trunks were removed. Both small and large potentials can be considered muscle responses to activity of centrally entering nerve fibres. The small potentials were probably identical with those observed by Hoyle & Lowy (1956). However, since these potentials did not seem to arise at random along the entire muscle, physiological evidence for a diffuse intramuscular ganglionic plexus is weakened. Micrographs by Bowden & Lowy (1955) were said to display intramuscular ganglion cells, forming a plexus around muscle fibres outside of nerve trunks. However, no internal structure was seen in these micrographs (Bowden, 1958). Abraham & Minker (1957) found no evidence of ganglion cells in molluscan adductor muscles. Deane & Twarog (1957) reported that characteristic basophilic ganglion cells are not demonstrable in the byssus retractor.

The intramuscular distribution of nerve endings is uncertain. Hoyle (1957) considered that Schmandt & Sleator (1955) demonstrated intramuscular conduction via multiterminal nerve fibres running the length of the byssus retractor muscle. Schmandt & Sleator had interpreted their data as implying true decremental conduction along muscle fibres. The apparent conduction velocity of neurally evoked potentials in this study was within the range of nerve conduction velocities reported in *Mya* (Horridge, 1958). However, intramuscular conduction via nerves is far from conclusively established. Electrical or chemical stimuli to the mid region produce maximum tension. In this same region maximum depolarization is measured after soaking in acetylcholine. Supposing multiterminal innervation, nerve endings may be more numerous in the mid region or the muscle membrane may respond actively only in this region, as at the spike-generating sites of the electroplax (Grundfest, 1957). Alternatively, nerve endings may be confined to this region.

The large spike-like potentials resembled muscle action potentials described by Fletcher (1937*a*), Prosser, Curtis & Travis (1951), and Schmandt & Sleator (1955). Both large and small spike-like potentials probably represent an active muscle response which may or may not

itself be propagated. While the small potentials may result from excitation of the muscle by random firing of single nerve fibres originating from major nerve trunks, the large potentials probably depend on synchronous activation by many nerve fibres.

Neurally evoked and spontaneous contractions were always preceded by large potentials. Bursts of small potentials alone never were associated with tension increments. Small-potential frequency was not consistently correlated with tension maintenance, nor was relaxation associated with alteration in small-potential frequency. With ganglia intact, small-potential frequency remained high, even during prolonged soaking in relaxing agents. A role of small potentials in sustaining contraction is therefore doubtful. Summarizing, the 'tetanus hypothesis' appears inadequate. A possible alternative is a true catch mechanism for which a molecular basis has been suggested recently by Johnson, Kahn & Szent-Györgyi (1959).

When the prolonged phase of contraction was abolished in 5-hydroxytryptamine, tension development was not blocked but actually increased (see Twarog, 1960). This, as well as absence of electrical activity during prolonged contraction, suggests independence of the tension-developing and tension-maintaining mechanisms.

SUMMARY

1. The innervation of the anterior byssal retractor muscle of *Mytilus edulis*, L. was traced. Excitor nerves were found to enter in the mid region of this long-fibred smooth muscle. A nerve–muscle preparation is described.

2. Electrical activity was recorded simultaneously with external electrodes placed at six sites along the length of the muscle while tension changes were followed.

3. 'Large' spike-like electrical potentials evoked by neural stimulation were 0·5–5·0 mV in amplitude and 80–140 msec in duration. These arose centrally and appeared distally with a delay corresponding to a conduction velocity of 25–50 cm/sec. The amplitude of these potentials decreased from the mid region to either end of the muscle.

4. Development of tension in response to neural and chemical stimuli was invariably preceded by 'large' potentials or depolarization of 0·5 mV or more. During rest and prolonged contraction no 'large' potentials were observed.

5. 'Small' spike-like potentials, 10–100 μV in amplitude, 60–100 msec in duration, were recorded during both rest and prolonged contraction. These appeared to be initiated centrally and conducted with loss in amplitude to the ends of the muscle. The 'small' potentials were not localized; potentials recorded distally were invariably represented at centrally placed

electrodes. The frequency of these potentials was maximal immediately following nerve section and regularly decreased as ganglia and nerve trunks were removed.

6. Bursts of 'small' potentials were never associated with tension increments; changes in frequency of 'small' potentials displayed no consistent relationship to tension maintenance. No decrease in frequency accompanied relaxation.

7. It is suggested that both 'small' and 'large' potentials are muscle potentials elicited by nerve fibres entering centrally. Synchronous activity as represented by the 'large' potentials appeared necessary in tension development. 'Small' potentials do not appear to play a part in sustaining tension. Since no electrical activity correlated with prolonged contraction has been detected, tension maintenance by a true 'catch' mechanism deserves consideration.

Sincere thanks must be expressed to Professor John H. Welsh at the Biological Laboratories, Harvard University, with whose help and guidance this work was initiated and carried out; to Dr Jerome Y. Lettvin, of the Research Laboratory of Electronics, Massachusetts Institute of Technology, who suggested the suction electrode and lent generously of critical advice and technical facilities; to Professor Alexander Forbes, Harvard University, who permitted the author to share the use of his ink-writing oscillograph for over a year, and to Dr F. A. Quadfasel and Dr W. J. Friedlander of the Boston Veterans' Administration Hospital, who lent the facilities of their research laboratory, and in particular the 8-channel electroencephalograph. This research was carried out during the tenure of a United States Public Health Service Research Fellowship of the National Heart Institute.

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EFFECTS OF ACETYLCHOLINE AND 5-HYDROXYTRYPTAMINE ON THE CONTRACTION OF A MOLLUSCAN SMOOTH MUSCLE

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(*Received* 11 *November* 1959)

Tension developed by the anterior byssal retractor of *Mytilus* in response to acetylcholine (ACh) decays very slowly after washing off the stimulating agent. 5-Hydroxytryptamine (5-HT) does not block tension development but relaxes the prolonged contraction. When 5-HT is present, tension is maintained only during stimulation (Twarog, 1954; Hoyle & Lowy, 1956). Acetylcholine-blocking agents prevent tension development, but even in high concentration do not affect tension decline in a contracted muscle (Twarog, 1959).

Acetylcholine and 5-HT have been detected in the byssus retractor by bioassay. Cholinesterase activity has been demonstrated (Twarog, 1954). Blaschko & Hope (1957) have found monoamine oxidase activity in the retractor muscle. Spectrophotofluorometric methods have revealed 5-HT in *Mytilus* ganglia (J. H. Welsh & M. Moorhead, unpublished).

Acetylcholine and 5-HT may be mediators released by nerves supplying the byssal retractor muscles. Details of the interaction of ACh and 5-HT on the excitability of the muscle membrane have therefore been studied. The influence of 5-HT and of certain acetylcholine-blocking agents on neural excitation of the muscle has also been examined.

METHODS

The equipment and basic procedure for mechanical and electrical recording and stimulation are described in detail in the preceding paper (Twarog, 1960).

In observations on membrane excitability and contraction maintenance, the muscle was dissected as described by Twarog (1954), teased down to a bundle about 1 mm in diameter and passed through a paraffin-lined slot in the chamber diagrammatically shown in Fig. 1. Tension and electrical potentials were simultaneously recorded on the ink-writer. Total demarcation potential, between the KCl-depolarized and sea-water segments, was 10–15 mV, never reaching the 18–25 mV observed with intact, unteased muscle. This may indicate injury in teasing. However, the potential was stable and bundles had the advantage that small-potential 'spontaneous activity' was virtually absent (Twarog, 1959).

When applying anodal and cathodal polarization only tension was recorded. The whole

muscle, cleaned from nerve, was used in the chamber shown in Fig. 1. At the shell end the chamber was drained and the muscle, suspended in air, made contact with a wick electrode. This, and the large loop of silver–silver-chloride wire in the sea-water chamber served as stimulating electrodes. The polarity of stimulus (e.g. anodal) refers to that of the sea-water chamber. In the nerve–muscle preparation tension was recorded simultaneously with the electrical potential between byssus and the region of nerve entry (Fig. 5).

RESULTS

The interaction of acetylcholine and 5-hydroxytryptamine: typical effects on membrane excitability and maintenance of contraction

When ACh was applied to a muscle previously untreated with drugs, it caused depolarization and development of tension (Fig. 1*a*). Each spike-like potential on the rising phase of depolarization was followed by a

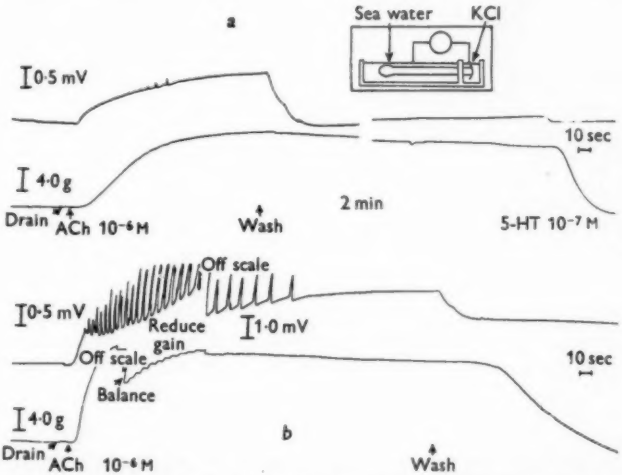

Fig. 1. (Inset) Recording of polarization changes in test segment of anterior byssal retractor muscle, with reference to KCl-depolarized segment. (*a*) Depolarization (upper channel) and contraction (lower channel) with ACh; relaxation by 5-HT. (*b*) ACh effects in the presence of 5-HT; records off scale, rebalanced and amplification reduced in electrical channel.

distinct small increment in tension. After washing, the muscle repolarized while tension remained high. When 5-HT 10^{-7} M was applied, the tension fell abruptly. If the 5-HT 10^{-7} M remained in the bath and ACh was then added (Fig. 1*b*), a striking change was seen. Large spike-like potentials and corresponding tension increments were prominent. The total tension developed was much increased. Tension was sustained after cessation of the rapid potentials until the muscle was washed, when rapid relaxation occurred. Clearly, 5-HT reduces the capacity of the muscle to sustain tension while potentiating electrical activity and tension development.

An interesting example of the relationship between phasic tension development and the maintenance of tension is seen in Fig. 2. This muscle had been left during the night at room temperature and responded to ACh with depolarization and a rhythmic discharge of potentials. Tension was developed and sustained with small tension increments apparent on the crest. (The response resembled that of the 5-HT-treated muscle of Fig. 1*b*.) When 5-HT was applied, as shown in Fig. 2, the rhythmic electrical activity was only slightly modified (increased amplitude, decreased fre-

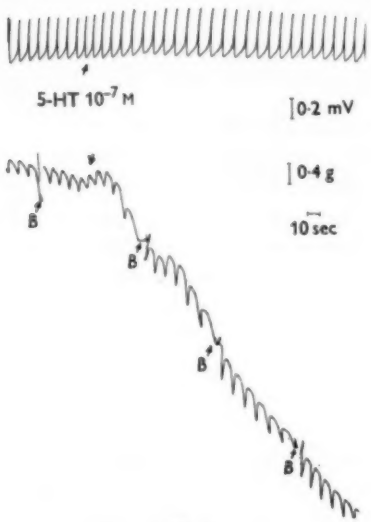

Fig. 2

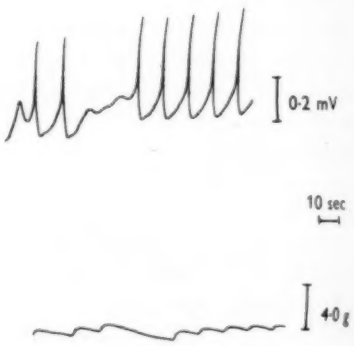

Fig. 3

Fig. 2. Electrical activity (above) and tension (below) in a rhythmically contracting muscle. Relaxation of maintained tension by 5-HT, off scale, rebalanced (*B*) and records appropriately aligned. Note constancy of rhythmical activity during relaxation.

Fig. 3. Electrical activity (above) and tension (below) in a rhythmically contracting muscle. Note failure to develop tension in absence of spike-like potential, lack of relaxation during cessation of discharge.

quency) and rhythmic contractions continued, while the muscle rapidly and fully relaxed. In Fig. 3 the same preparation as in Fig. 2 is seen during the maintained contraction (before 5-HT). Electrical activity was occasionally irregular. A small, rounded electrical potential appeared at time intervals appropriate to the original rhythm, when the spike was absent, but no tension increment accompanied this potential. It is noteworthy that relaxation following spike failure was negligible compared with the relaxation in Fig. 2 which occurred during continuous spike activity.

Effects of anodal and cathodal polarization on development and maintenance of tension

Sensitivity to anodal and cathodal polarization differed during spike-coupled rhythmic tension changes and maintained tension, as is seen in Fig. 4. Here an anodal direct current pulse had no effect when applied during the last stages of tension decay following a long cathodal pulse. A long cathodal pulse at this time led to a prolonged contraction. Brief repetitive cathodal pulses had no effect, while brief anodal repetitive pulses of the same voltage led to relaxation after a small tension increase. (The effectiveness of brief anodal pulses was noted by Fletcher (1937), and may be significant

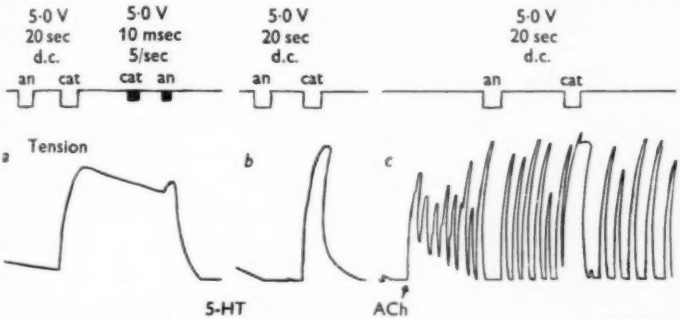

Fig. 4. Effects of anodal (an) and cathodal (cat) polarization on tension. (*a*) Muscle in last stages of maintained contraction. Anodal and cathodal direct current, single long pulses, then brief repetitive pulses. (*b*) Muscles relaxed in 5-HT; long anodal and cathodal pulses. (*c*) Muscle rhythmically contracting in ACh plus 5-HT; long anodal and cathodal pulses.

in synchronizing responses.) A cathodal pulse again led to prolonged tension in this preparation, which was relaxed by 5-HT (not shown). In the presence of 5-HT, a long anodal pulse was again without effect, while a cathodal pulse led to a larger tension increase which relaxed at the cessation of stimulation. In the continued presence of 5-HT, the response to ACh was a series of rhythmic contractions. In the rhythmically active muscle, anodal direct-current pulses inhibited contraction and decreased the tension level, while cathodal polarization led to a contraction which relaxed at the end of stimulation.

Effects of acetylcholine-blocking agents on neural excitation of the muscle

Stimuli of 1 msec duration were applied through the nerve at the rate of 1/sec, as in Fig. 5. The upper, control, record showed facilitation of contraction with repetition of identical stimuli. After soaking the muscle for 3 min in 10^{-3} M banthine, the contractile response was blocked and the

electrical response became smaller and rounded in shape. Other ACh-blocking agents, including atropine and benzoquinonium, similarly altered the large spike-like potential and blocked the contractile response to neural excitation at concentrations between 10^{-4} and 10^{-3} M. In concentrations of 5-HT up to 10^{-4} M, the electrical response was not altered nor was contraction blocked, although an increase in threshold was noted in some experiments.

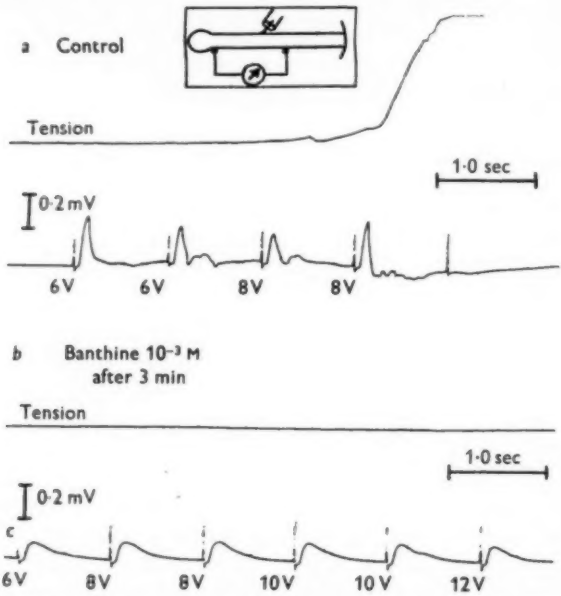

Fig. 5. (*a*) Untreated muscle; electrical response below, tension above; note facilitation with 8 V shock. (*b*) After banthine 10^{-3} M; tension (above) blocked; electrical response (below, reduced) prolonged. Inset, placement of stimulating and recording electrodes.

DISCUSSION

Block of neural excitation by ACh-blocking agents, as observed here, supports a hypothesis of excitatory transmission by ACh. It has been argued by Hoyle & Lowy (1956) that 5-HT cannot be a transmitter, since its action was not easily reversible in their experiments. In the present study the effects of concentrations of 5-HT which fully relax the muscle were reversed within minutes by washing. The apparent discrepancy may be due to the higher concentrations used by Hoyle & Lowy. Enzymic destruction of 5-HT in the byssus retractor has been demonstrated by Blaschko & Milton (1959).

Rhythmic contractions in combined ACh and 5-HT have been seen in

other molluscan smooth muscles (Hill, 1958; Fänge & Mattisson, 1958). In the present study rhythmic spike-like potentials preceded rhythmic contractions. Anodal polarization inhibited these contractions. In guinea-pig smooth muscle, spike discharge and tension are suppressed by anodal polarization (Bülbring, 1956). It appears probable that rhythmic contractions of the byssus retractor arise from electrical activity of the muscle membrane.

The prolonged contraction after ACh is apparently not associated with electrical activity (this paper and Twarog, 1960), nor was prolonged contraction relaxed by anodal polarization. This suggests that the elements which maintain tension may be independent of membrane polarization and electrical activity, once tension has been developed. Johnson, Kahn & Szent-Györgyi (1959) suggested that the tension-developing and -maintaining systems have separate molecular bases.

The action of 5-HT somewhat resembles β inhibition in crustaceans. As in studies by Hoyle & Wiersma (1958) on crustacean muscle, large mechanical effects accompanied very small changes in membrane polarization. According to Hoyle & Wiersma an inhibitory transmitter might block excitation–contraction coupling or could cause a change in permeability to ions important in contraction, such as Ca^{2+}. If tension maintenance is independent of the membrane activity which leads to tension development, a relaxing mechanism specific to the tension-maintaining system must exist. If continued activation is not involved in tension maintenance, then uncoupling is a possible but unnecessary postulate.

SUMMARY

1. Relaxing concentrations of 5-hydroxytryptamine (10^{-7} M) altered the response of the byssus retractor of *Mytilus* to acetylcholine. In the presence of 5-hydroxytryptamine, acetylcholine evoked a succession of spike-like electrical discharges. Total tension developed was greater than in the absence of 5-hydroxytryptamine but the contraction was not sustained following removal of acetylcholine.

2. Anodal polarization inhibited rhythmic contractions but did not accelerate the slow decline of tension after acetylcholine.

3. Acetylcholine-blocking agents (banthine, atropine and benzoquinonium, 10^{-4}–10^{-3} M) prevented the contractile response to neural stimulation and reduced the electrical response. 5-Hydroxytryptamine (as high as 10^{-4} M) did not interfere with neural excitation.

4. While firing of spike-like potentials appears necessary for tension development, tension following stimulation may be maintained by non-discharging elements. Possible mechanisms of relaxation by 5-hydroxytryptamine are discussed in this connexion.

The guidance and encouragement of Professor John H. Welsh, in whose laboratory this work was carried out, is acknowledged here with most sincere thanks. This research was carried out during the tenure of a United States Public Health Service Research Fellowship of the National Heart Institute.

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EFFECTS OF ADRENALINE ON THE ISOLATED UTERUS OF THE CAT

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(*Received* 13 *November* 1959)

Over fifty years ago Dale (1906) showed that the response of an isolated strip of cat uterus to added adrenaline varies with the state of the animal in relation to pregnancy. Uteri from virgin cats or non-pregnant cats in oestrus or in anoestrus are inhibited by adrenaline. If there is spontaneous activity it ceases; if there is high tone it relaxes; if there is no movement and the muscle is relaxed the quiescent condition is not disturbed and may be prolonged by adrenaline. If the virgin cat is injected subcutaneously with a preparation of oestrogenic hormone the uterus enlarges, becomes congested and when isolated is frequently found to be active, but the response to added adrenaline is unaltered. If the cat from which it is taken is pregnant, as Dale showed, the uterine muscle responds to adrenaline with contraction. This change in the nature of the response develops shortly after pregnancy begins and progresses with the gain in progesterone control. It is lost again with involution of the uterus post partum. Similarly, the altered response to adrenaline may be induced by injecting an oestrous cat with progesterone and it develops with proliferation and other evidences of pregnancy hormonal action.

We have explored the possibility of altering the response to adrenaline of an isolated strip of cut uterus by preparations from tissues of the same or other female cats.

METHODS

Female cats were collected in pairs as follows: (*a*) one pregnant one non-pregnant, both parous, between February and September; (*b*) neither pregnant, both parous, at any time of the year: these cats were given 0·5 mg stilboestrol daily for 10 days by subcutaneous injection; then one was injected with stilboestrol, the other with progesterone 2 mg/day for a further 10 days. (*c*) One pregnant, one virgin, 4–6 months of age, reared in captivity. In some cases the virgin cat was injected for 10 days with stilboestrol as above. (*d*) One virgin, reared in captivity for 6 months, one pseudo-pregnant, parous, injected with stilboestrol and then progesterone as in (*b*).

The cat was lightly anaesthetized with ethyl chloride spray and exsanguinated, and

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the uterus (without ovaries) removed. A strip of the uterus was mounted in Locke's solution at 37° C., bubbled with 95 % O_2 + 5 % CO_2, and its tone and movement were recorded with a frontal-writing lever. The size of bath was usually 10 ml., but 30 ml. for two specimens together. The response to adrenaline 10^{-6}–10^{-8} was recorded under certain sets of conditions detailed below. Extracts were made initially by grinding tissue with washed powdered glass, latterly in homogenizers, in 2 ml. Locke's solution/g wet tissue. Skeletal muscle, lung, liver and uterus were prepared in this way and added in varying amounts to the bath for 10–15 min before testing with adrenaline. Some extracts were boiled and cooled and the filtrate used. Progesterone 2 mg in oil was emulsified with warm Locke's solution 10 ml. and concentrate of Tween 80 0·3 ml. and put into the bath containing oestrous uterus for 1 hr before testing with adrenaline. The combinations of isolated uteri and extracts examined were as follows: (*a*) virgin; naturally oestrous parous non-pregnant; oestrogen-injected virgin; oestrogen-injected anoestrous parous—alone; (*b*) the same, with, in the bath, a 1–2 g piece of skeletal muscle, liver, gut or lung; (*c*) the same with, in the same 30 ml. bath, a strip of pregnant or pseudopregnant uterus as in (*e*). The tissues were suspended from separate levers which had no contact, and remained for 2–3 hr together before testing with adrenaline; (*d*) the same, with, in the 10 ml. bath, homogenate of skeletal muscle, liver, lung or of uterus equivalent to 1 g of tissue. The homogenized uterus was either (1) oestrous non-pregnant (natural or injected with stilboestrol); (2) pregnant; (3) pseudopregnant adult (injected with stilboestrol and then progesterone). The extract was added and a variable time allowed to elapse before adding adrenaline. The bath might contain atropine sulphate 10^{-8} or mepyramine maleate 10^{-8}. To one piece of an oestrous uterus, the extract of progesterone-proliferated uterus was added as described, while to another piece of the same oestrous uterus ergotamine tartrate 10^{-5} or phenoxybenzamine 10^{-6} was first administered for 15 min; (*e*) a strip of pregnant uterus cleaned of its endometrium, or of pseudopregnant uterus (progesterone-injected cats)—alone.

RESULTS

When the isolated uterus of a parous cat taken in the spring season (natural oestrous) was treated with adrenaline, spontaneous rhythm ceased and tone, if present, was relaxed. The sensitivity of the tissue was not great and a concentration of 10^{-7} was often needed. A similar response was obtained with the uterus of oestrogen-injected cat which showed much spontaneous activity. Virgin cat uterus often had no spontaneous rhythm and little tone, in which case it remained apparently unaffected by added adrenaline. The patterns of spontaneous activity varied widely, but in no case did adrenaline 10^{-6} fail to produce or maintain a prolonged relaxation and quiescence. The presence in the bath of a piece of liver, lung, gut or skeletal muscle did not modify the response of the non-pregnant uterus to adrenaline.

If the uterus was taken from a pregnant cat, especially if late in term, and the contents and lining were removed, addition of adrenaline 10^{-8} caused spontaneous rhythm to speed up or start; tone developed or increased. The pseudo-pregnant uterus reacted similarly and both these tissues were more sensitive to adrenaline than is non-pregnant uterus; 10^{-8} almost always produced a clear response and 10^{-6} invariably produced spasm.

If a larger strip of pregnant cat uterus was mounted in the same bath with a smaller strip of non-pregnant uterus from a parous cat, and left for 2–3 hr, various changes in tone and spontaneous rhythm of one or other specimen occurred. Very often both became quiescent. If adrenaline was then added the pregnant uterus gave a motor response but the non-pregnant uterus also made a motor response—a change or reversal of the response expected. This change has been reported and illustrated by Graham & Gurd (1959). Washing restored the customary inhibitor response to the non-pregnant uterus. Similarly, if the larger tissue was from a pseudo-pregnant cat (progesterone-treated) and the smaller strip from an oestrogen-injected cat, they both responded to added adrenaline with a contraction. If the oestrous portion was showing spontaneous activity this increased in rate and diminished in extent; if it was fully relaxed it showed a rise in tone and movement began. These reactions, taken from different specimens on separate occasions, are shown in Fig. 1. Only pregnant or pseudo-pregnant uterus will bring about the complete reversal of the response to adrenaline in the uterus of an oestrogen-injected cat. Other tissues or non-pregnant oestrous or anoestrous uterus have no effect, nor does the addition to the bath of progesterone in oil. If the non-pregnant segment was from an immature virgin cat the pattern of spontaneous activity altered but there was no 'reversal' of the action of adrenaline.

If a piece of uterus from a pregnant cat or from a cat injected as described with progesterone is macerated or homogenized with cold saline and centrifuged or filtered and the supernatant or filtrate taken, this may be termed a simple extract. When a portion of simple extract was added to a bath containing a piece of non-pregnant parous uterus and adrenaline, in an amount which previously elicited an inhibitor response added after some 10–15 min, the response was an excitor one. Extracts of most tissues have an oxytocic effect on isolated non-pregnant parous cat uterus (Collip, 1922). If the extracts were made from liver, lung, gut, muscle, or non-pregnant uterus, the reaction to adrenaline of the isolated uterus to which the extracts were added might be antagonized during the oxytocic phase but not reversed (see Fig. 2, Nos. 3 and 4). Simple extracts of pregnant or progesterone-proliferated uterus are also oxytocic but in addition they 'reverse' the response to adrenaline. The reversal can be demonstrated while the oxytocic effect is in progress, after it has passed off (Fig. 2, No. 1) or before it has developed (Fig. 2, No. 2) as there is usually a latency. If the oestrous uterus was in rhythmic movement this was speeded up, or increased in excursion; if it was quiescent it contracted and rhythmic activity followed.

A variable minimal amount of extract was needed before reversal occurred and increasing doses had to be added in order to reverse any

given piece of non-pregnant tissue. Not all extracts were effective in doses such that the oxytocic effect did not cause prolonged spasm and obscure the result. The oxytocic effect was unaffected by boiling the extract, but was reduced if mepyramine maleate or atropine sulphate 10^{-8} was first added to the bath. Reversal was not affected. Prolonged soaking of

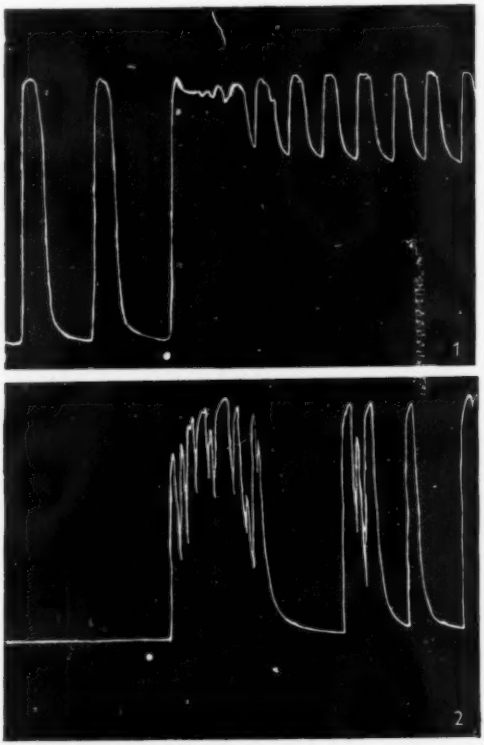

Fig. 1. Isolated strips of uterus from two cats in oestrus. With each, in the same bath, there has been a large portion of uterus from a cat injected with progesterone. The response to added adrenaline of the non-pregnant uterus is now reversed. (1) Spontaneous contractions speeded and tone increased; (2) quiescent uterus roused to activity.

the oestrous uterus in extract rendered it insensitive to stimulants. This is a phenomenon unrelated to 'reversal', but may obscure results. The 'reversal' effect, once demonstrated, was removed by washing, but some accumulation must occur, as lesser amounts of the same extract were effective in consecutive trials on the same tissue. Prior addition of ergotamine tartrate or of phenoxybenzamine to oestrous tissue prevented the development of reversal to adrenaline by extract of progesterone-pro-

liferated uterus. If the non-pregnant uterus was from a virgin cat, extract of pregnant uterus antagonized the response to adrenaline but did not reverse it. Extract of progesterone-proliferated uterus also had an oxytocic effect, but brought about a true reversal to adrenaline if the virgin

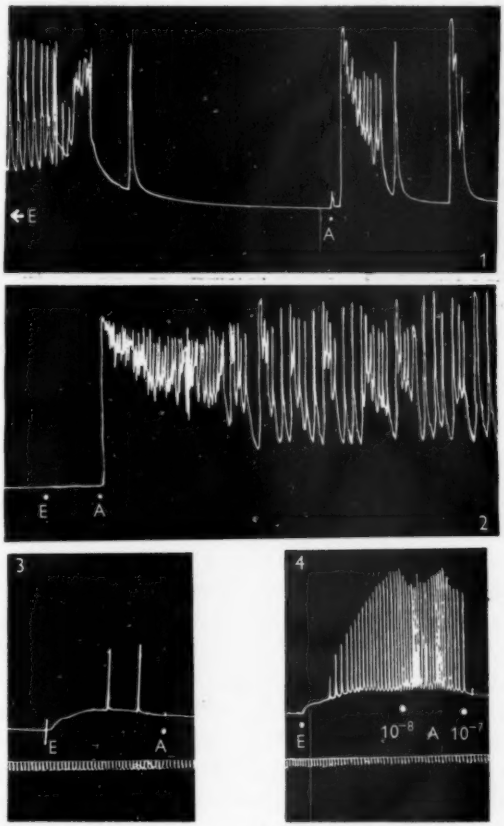

Fig. 2. Isolated strips of uterus from cats in oestrus. The effects of adding extracts of tissue and then adrenaline. (1) and (2) extract of 1 g uterus of cat treated with progesterone; action of adrenaline reversed (1) after and (2) before onset of oxytocic effect of extract. (3) Extract of 1 g skeletal muscle. (4) Extract of 1 g non-pregnant uterus; action of adrenaline not reversed. Time marker, 30 sec.

cat was well developed (6 months of age; 3 experiments) but not if it was very immature (3 experiments) and inactive when isolated. The presence of a large piece of non-pregnant cat uterus in a bath with a small piece of pregnant cat uterus has no effect on the response of the latter to added adrenaline.

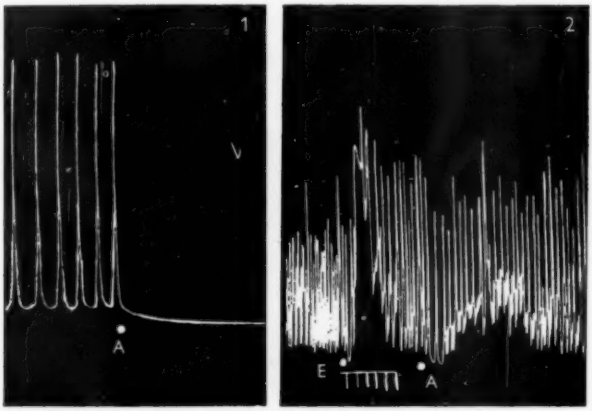

Fig. 3. Isolated strip of uterus from virgin cat (6 months old). (1) Adrenaline inhibits activity. (2) After the oxytocic effect of extract of 1 g uterus from a cat treated with progesterone has passed off adrenaline had a motor action. Time marker, 1 min.

DISCUSSION

McSwiney & Brown (1926) studied the response of several tissues, including rabbit stomach and uterus, to added adrenaline in various conditions of tone of the preparation. They concluded that 'the mechanism controlling the augmentor and inhibitor response to adrenaline may be present in all preparations of smooth muscle capable of active contraction and relaxation'. As far back as 1919 Cow examined non-pregnant uterus from guinea-pig. Normally he found this muscle inhibited by added adrenaline but after a soak in pituitrin (an extract of whole gland which caused a spasm) the muscle contracted to added adrenaline. Collip's work (1922) is also important. He found that a watery extract of most tissues was oxytocic. 'Extracts, in addition to causing intense stimulation of the uterus antagonized the inhibitory action of adrenaline on such uteri as are normally inhibited by this latter substance, as in the case of the rat and guinea-pig (both virgin and gravid) and the virgin rabbit and dog'. He does not mention or illustrate a true reversal of the response to adrenaline but speaks of 'an instance of certain tissue constituents acting selectively upon the inhibitory nervous apparatus of a tissue irrespective of whether it is of the sympathetic or parasympathetic type'.

The present experiments are concerned with contraction in isolated uterus rather than with changes in tone. The results imply that something is present in pregnant or progesterone-proliferated cat uterus which can be extracted by water and which can diffuse in a bath. This substance,

which may be a water-soluble, quick-acting form of pregnancy hormone, does not behave like progesterone. It is capable of penetrating oestrous uterine muscle and altering it so that the response to adrenaline becomes a motor one. Immature muscle which may not have experienced the influence of oestrogen is not affected. The site of action is such that anti-adrenaline compounds (ergot alkaloid or phenoxybenzamine) prevent it.

SUMMARY

If non-pregnant cat uterus is treated with an extract of pregnant cat uterus the action of adrenaline on it is reversed. This effect is prevented by phenoxybenzamine.

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FUNCTIONAL ANALYSIS OF GROUP III AFFERENT FIBRES OF MAMMALIAN MUSCLES

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It is generally accepted that the myelinated afferent fibres of some muscle nerves fall into three groups according to the diameter of their nerve fibres (Lloyd, 1943; Lloyd & Chang, 1948; Rexed & Therman, 1948; Hagbarth & Wohlfart, 1952), a grouping also borne out by electrophysiological studies (Hunt, 1954). The fibres of Group I (12–20 μ) arise from muscle spindles and Golgi tendon organs (Hunt & Kuffler, 1951; Hunt, 1954). Those of Group II (4–12 μ) also arise from muscle spindles (Merton, 1953; Hunt, 1954) but not from tendon organs (Hunt, 1954). Further, recent evidence indicates that the Group II fibres probably arise from the secondary endings of the muscle spindle (Cooper, 1959).

So far nothing is known about the endings of Group III afferent fibres (1–4 μ), although there is considerable information about the distribution of fibre diameters within this group in different muscle nerves (Lloyd & Chang, 1948; Rexed & Therman, 1948; Hagbarth & Wohlfart, 1952). As will become clear from the present paper, this gap in our knowledge of the sensory innervation of muscles was apparently due to the difficulty in stimulating some of the Group III endings and also to certain experimental procedures followed by previous workers. The experiments to be described in this paper were therefore, at first, specifically designed to elucidate the behaviour of these Group III endings, the basic procedure being to isolate a Group III afferent fibre, i.e. one with a conduction velocity below 24 m/sec, and then to determine how its ending could be stimulated. The results of this procedure have revealed that most Group III afferent fibres end in pressure receptors, i.e. receptors that are stimulated by local pressure but not by stretching the muscle or by asphyxia. Hitherto little attention has been paid to these receptors owing to the general belief that there are few of them in muscles (Hagbarth & Wohlfart, 1952). However, the recent

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histological studies of Barker (1959), showing that there are significant numbers of so-called pressure receptors in the rectus femoris, should serve to focus attention on them.

METHODS

Experiments were carried out on adult cats anaesthetized with chloralose (70–80 mg/kg) after preliminary induction with ether. The arrangements for fixing the left hind limb, stimulating nerves and recording nerve impulses from afferent fibres were identical with those used previously (Paintal, 1959*a*).

In the earlier experiments filaments were dissected from the lateral gastrocnemius-soleus nerve and the medial gastrocnemius and tibial nerves were cut. Nerve action potentials were recorded monophasically from the peripheral end of the cut filament. When recording impulses from afferent fibres of the tibialis anterior, the superficial peroneal and extensor digitorum longus nerves and the deep peroneal nerve distal to its branch to tibialis anterior were cut. In these experiments the stimulating electrodes were placed under the nerve close to the entry of the nerve into the tibialis anterior muscle. Nerve impulses from tibialis anterior endings were recorded in filaments dissected from the deep peroneal portion of the common peroneal nerve. This proved to be convenient because it provided adequate conducting distance over which to measure the conduction velocities of fibres. Although by this procedure fibres destined for regions other than tibialis anterior, e.g. extensor digitorum longus, were not eliminated, the fact that a particular fibre belonged to the tibialis anterior nerve could always be established by observing the characteristic impulse evoked by stimulating the nerve near its entry into the muscle or by the effects of antidromic stimulation (Paintal, 1959*b*). This was necessary, although the nerves to all regions other than tibialis anterior were cut, because of possible vicarious stimulation of cut nerve fibres. The peroneus longus and extensor digitorum longus muscles were cut from their origins and separated from tibialis anterior muscle for a distance of 2–4 cm to expose the point of entry of the tibialis anterior nerve into the muscle.

Conduction velocities of individual afferent fibres were determined by the methods and criteria described previously (Paintal, 1953). The likely sources of error were also similar to those described in that paper, and as before no arbitrary allowance of 0·1 msec was made for setting-up time at the stimulating electrodes (Blair & Erlanger, 1936).

The conduction velocities of most of the fibres that could be activated by mechanical stimuli were established by criteria mentioned above (i.e. Paintal 1953), but in some such proof could not be obtained. In the latter even the simpler though less reliable criterion of correlating the appearance of a component of the compound action potential with the simultaneous appearance of evidence of antidromic invasion of the sensory ending, as indicated by re-setting of the rhythm of the ending (Iggo, 1958), was of no help, because of the absence of a slowly adapting train of impulses in the fibres examined in the present investigation (see below). In such fibres strong evidence of identity of a fibre with a particular conduction velocity with that yielding the impulses on natural stimulation was obtained as follows: The cat was curarized and the stimulus to the nerve adjusted so that it was just subthreshold for the fibre suspected to be the one activated by natural stimulation (spike at arrow, Fig. 1*A*). With this stimulus strength, just sufficient to produce the compound action potential appearing with a latency of 1–1·5 msec in Fig. 1*A* (but not the spike at arrow), the nerve was stimulated repetitively at about 90/sec and the natural stimulus applied to the muscle, as in Fig. 1*B*. If impulses appeared as in Fig. 1*B* it was clear that they did not belong to one of the fibres in the faster-conducting component. The stimulus was then increased to include the slower fibre under examination and if the impulses did not appear it was concluded that the fibre activated by natural stimulation was identical with that yielding the specific slower component. This test was very convenient

because the potentials of the fibres conducting below 24 m/sec stood out clearly from the faster compound action potential as discrete individual spikes (Fig. 1*A*).

The temperature of the paraffin pool was measured with a mercury thermometer and kept as close to 37° C as possible. This was not difficult because the room temperature at which most of the conduction velocities were determined was about 37° C. The intra-abdominal temperature was used as a measure of deep body temperature. In those instances where the pool temperature deviated from 37° C a temperature correction in the conduction velocity was made by using a Q_{10} of 1·3 for *in situ* conditions, as in the cervical vagus (Paintal, 1953).

The tension of the muscle concerned was measured with a strain gauge (Statham) and d.c. amplifier and recorded on one channel of the oscilloscope.

Flaxedil (May & Baker) or tubocurarine were used to produce neuromuscular block whenever required.

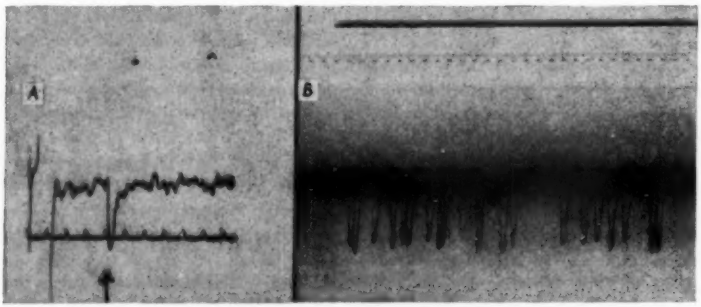

Fig. 1. Records showing how the identity of a particular sensory fibre stimulated by an electrical stimulus with that activated by natural stimulation was demonstrated in some experiments. *A* is a single sweep showing an early fast-conducting compound action potential followed by a single spike in a slowly-conducting fibre (conduction velocity, 14·1 m/sec) at arrow. In *B* the strength of the stimulus was reduced a little so that it did not stimulate the slow fibre but it elicited the entire early compound action potential. The sweep was set 'free running' and this compound action potential was kept out of view so that it did not mask the impulses elicited by pressing the muscle at signal in the continuous record shown in *B*. The spikes in *B* are similar to that at arrow in *A*. When the electrical stimulus was increased to stimulate the slow fibre as well, the naturally evoked impulses shown in *B* did not appear. From above downwards in *B*, signal, time marker 1/10 sec, impulses.

RESULTS

Receptors in lateral gastrocnemius and soleus

Initially, the main aim was to isolate Group III afferent fibres with conduction velocities below 24 msec. While doing so it was necessary to ensure that fibres having an 'early discharge' (see Masland & Wigton, 1940; Lloyd, 1942; Hunt & Kuffler, 1951; Granit, Pompeiano & Waltman, 1959; Cooper, 1959) were not mistaken for slowly-conducting fibres. As is shown in Fig. 2, this was achieved by placing a second stimulus of greater

intensity at a proper interval before the appearance of the slow impulse. If the slow impulse belonged to the 'early discharge' it was delayed or abolished (Fig. 2*A*, *B*) because of antidromic depression of excitability (Matthews, 1933; Paintal, 1959*b*). The second stimulus had to be of greater intensity than the first in order to avoid possible relative refractoriness of the nerve fibres following the first. This procedure also avoided inclusion of impulses arising out of repetition at the stimulating electrodes.

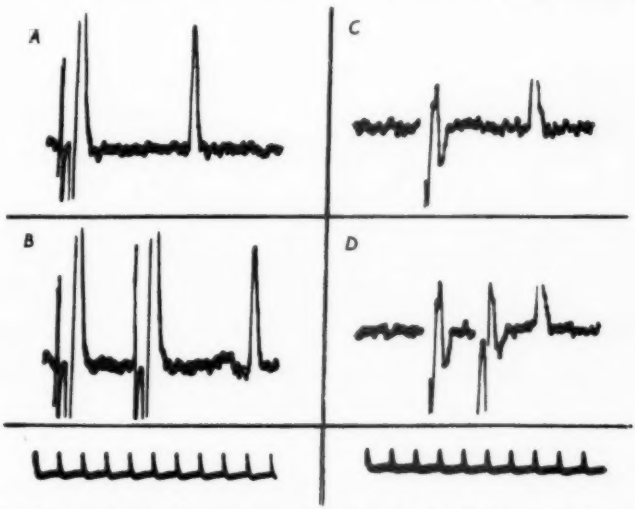

Fig. 2. Sweeps showing how an impulse of an 'early discharge in *A* was distinguished from an impulse belonging to a slowly-conducting fibre (conduction velocity, 10·2 m/sec) in *C*. Application of a second stimulus in *B* delays the 'early discharge' impulse owing to antidromic depression, but leaves the slowly-conducted impulse unaffected in *D*. Time marker, msec.

The fact that any fibre with conduction velocity below 24 m/sec was sensory in function was regarded as established if natural peripheral stimuli such as stretch or pressure initiated impulses in it characteristic of responses of sensory receptors. If impulses could not be initiated then doubt about the sensory identity of the fibre remained *only* if it had a conduction velocity above 15 m/sec, because the lower limit of conduction velocities of efferent fibres (i.e. arising from the ventral roots) is 15 m/sec (Kuffler, Hunt & Quilliam, 1951; Hunt & Paintal, 1958). In this investigation only three such fibres from lateral gastrocenmius and soleus and one from tibialis anterior were met with.

Responses of Group III fibres. Out of 31 Group III afferent fibres isolated from the lateral gastrocnemius-soleus nerve 6 could not be stimulated by any type of mechanical stimulus such as pulling, pressing, squeezing or

prodding the muscle. Two fibres were stimulated by pulling the muscle, the discharge being characteristic of muscle stretch receptors. The response in one of these during muscle twitch indicated that it originated from the muscle spindle, presumably from a secondary ending. The conduction velocities of these two fibres were respectively 18 and 21 m/sec.

The majority of Group III afferent fibres, 23 out of 31, were stimulated by local pressure but not by stretching the muscle (Figs. 3, 8). Most of them did not yield even one impulse on pulling the muscle rapidly. Such

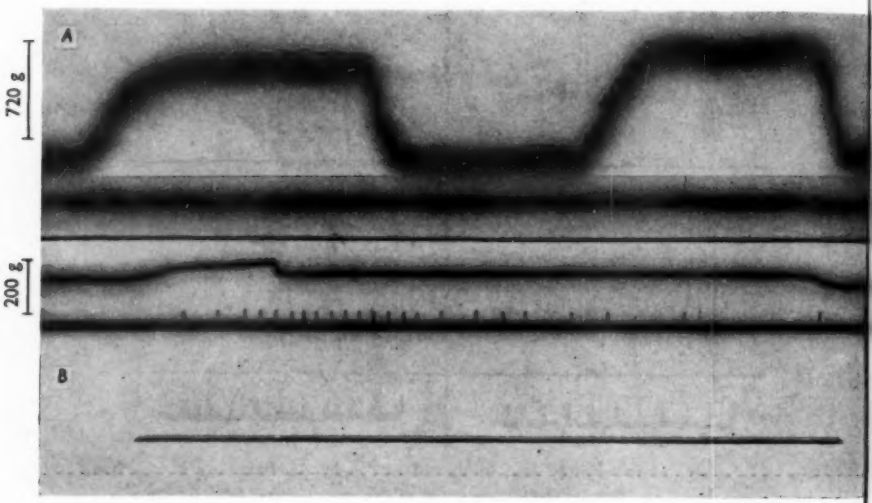

Fig. 3. Responses of a pressure receptor in lateral gastrocnemius-soleus muscle. *A*, shows that stretching the muscle to over 700 g did not stimulate the ending, whereas pressing the muscle in *B* yielded a train of impulses. The rise in muscle tension in *B* is due to pressing the muscle but it does not indicate the amount of pressure applied. From above downwards in *A* and *B*, record of muscle tension, impulses in a filament and in *B*, signal and time marker 1/10 sec. Conduction velocity of afferent fibre was 14·1 m/sec.

endings may therefore be regarded as pressure receptors and will be referred to as such in this paper. In these experiments the muscle was stretched sufficiently to raise the tension by from over 400 to 700 g (Figs. 3, 8); in some fibres this yielded one impulse, in others two or three impulses in some of the trials. From such observations it was clear that the fibres were not connected to stretch receptors, considering that the threshold of most stretch receptors is less than 350 g (Matthews, 1933; Hunt & Kuffler, 1951), although the threshold of some of Matthews's B endings was as high as 700 g.

The response to local pressure varied in different receptors not only quantitatively, but also in the type of mechanical stimulus that stimulated them. Thus while some were stimulated by pressing the appropriate region of the muscle gently with a glass rod, others could be stimulated only by squeezing the muscle between finger and thumb. Again, the direction in which the muscle was pressed or squeezed was important. Also, sometimes the receptors which were stimulated by pressing with a glass rod were poorly stimulated by squeezing the muscle. Owing to such variations it was not feasible to use any single device for applying pressure.

A little less than half the pressure receptors isolated could be stimulated consistently each time local pressure was applied at the proper place. At the other extreme there were receptors that could be stimulated in only 20 % of the trials under the most favourable circumstances. The remaining receptors fell in between these two extremes.

The stimulus threshold was difficult to assess in those receptors which could only be stimulated by pressing or squeezing the muscle manually. However, a rough estimate of the pressure exerted was made by noting the pressure recorded by an algometer (Baird & Tatlock) when pressure of the same order was applied to it. The threshold estimated roughly in this way varied from 300 g to 2 kg/cm.^{2} Those receptors that could be stimulated by pressing locally with a blunt point allowed a more precise measurement of the threshold to be made, as direct pressure could be applied by the blunt point of the strain gauge which had an area of about 16 mm^2. The threshold pressure registered by the strain gauge varied in different receptors from 25 to 200 g. The threshold varied somewhat in each trial and also with the direction of application of pressure. The receptors were apparently more easily stimulated if the muscle was not stretched initially, but owing to the variation in the responses mentioned the precise influence of stretch on stimulus threshold was not studied.

Most of the pressure receptors adapted rapidly, the discharge ceasing in about 0·5–2 sec after its onset. About a third of them adapted more slowly (Figs. 3, 8) and there were only a few which showed a truly slowly-adapting type of discharge.

The peak frequency of discharge also varied considerably from one fibre to another, the lowest value being in the range of 2–10 impulses/sec and the highest 100–150 impulses/sec. Such peak frequencies were averaged over 0·1 sec at high frequencies and over 0·5 sec in the low-frequency range. Attempts to raise the peak frequency of the receptors firing at a low frequency were seldom successful. These observations contrast with the responses of stretch receptors, in which the peak frequency may reach 500/sec (Matthews, 1933).

An attempt was made to determine the range of conduction velocities

over which the fibres of pressure receptors were distributed. This was achieved by reversing the sampling procedure, so that fibres in which impulses were aroused by pressing the muscle, but not by stretching it,

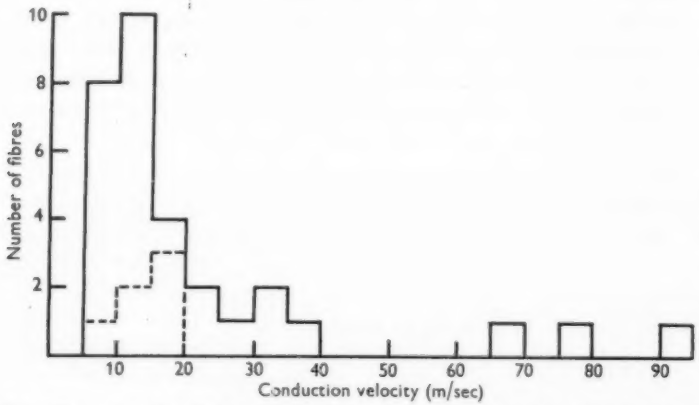

Fig. 4. Frequency distribution of conduction velocities of afferent fibres connected to pressure receptors in lateral gastrocnemius and soleus muscles. Interrupted line, distribution of fibres that could not be stimulated by any type of mechanical stimulus.

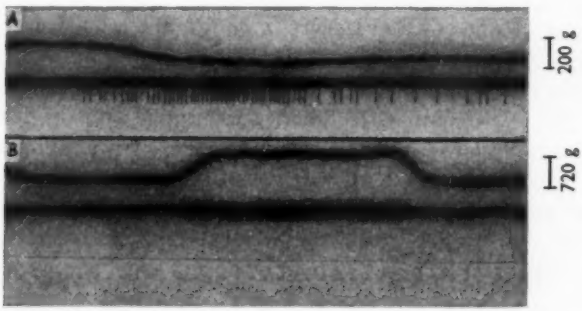

Fig. 5. Response of a pressure receptor of lateral gastrocnemius-soleus connected to a Group I afferent fibre. *A* shows that pressing the muscle locally with the tip of the strain gauge yielded a train of impulses, whereas stretching the muscle from 0 g initial tension to over 700 g did not stimulate the receptor, although it stimulated 2 stretch receptors which were not stimulated by local pressure in *A*. From above downwards in *A* and *B*, record of strain gauge, impulses in fibres; and in *B*, time marker 1/10 sec.

were isolated first and then the conduction velocities of such fibres were determined. The results of these experiments showed that there was a wide range of conduction velocities extending from 6 to 91 m/sec. How-

ever, there are apparently few fast-conducting fibres, because only 4 fibres with conduction velocities of > 55, 66, 76, and 91 m/sec were encountered in the lateral gastrocnemius-soleus nerve. The distribution of the conduction velocities of these fibres have been plotted in Fig. 4. It is clear from these results that pressure receptors with characteristics described above have afferent fibres distributed in all three groups, but the majority of them are in Group III, the peak being in the 10–15 m/sec range. In this

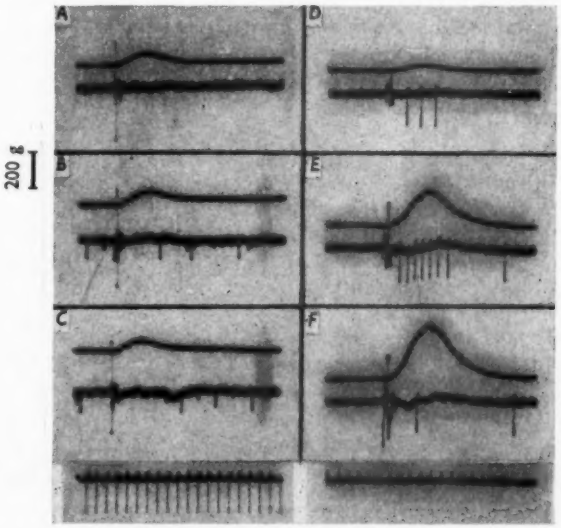

Fig. 6. Effect of muscular contraction on pressure receptors of lateral gastrocnemius-soleus. In *A*, muscle twitch without local pressure had no effect; in *B* and *C* muscle twitch with applied local pressure gave no response during contraction. Conduction velocity of afferent fibre was 33·6 m/sec. In records *D*, *E*, and *F*, which are from another fibre (conduction velocity 23 m/sec), local pressure applied with a glass rod was kept constant but the strength of muscular contraction was increased from *D* to *F*; note optimal contraction in *E*. Time marker, 10 msec. Calibration at left applies only to *A*, *B*, *C*.

series only 4 fibres in the Group II range were encountered, but it is felt that with better sampling procedure perhaps a slightly greater number will be found in Group II. All the pressure receptors had the characteristics of Group III pressure receptors described above, i.e. they were unaffected by stretching the muscle to over 700 g but were stimulated by local pressure. The discharge of the endings with Group I fibres was in most cases slowly-adapting and the threshold seemed lower, these being perhaps the only significant differences from those of Group III (Fig. 5).

Effect of muscle twitch. The effect of muscular contraction was observed

in only 13 out of 24 pressure receptors. This was because neuromuscular block which was often required for identification did not permit similar observations to be made in the remaining receptors. Five out of the 13 examined were unaffected by muscular contraction with or without applied external stretch. The responses of the remaining 8 were rather unimpressive and varied in the same ending (Fig. 7*A*). Five of the latter showed an 'early discharge' somewhat characteristic of that seen in muscle spindles and tendon organs (Hunt & Kuffler, 1951; Granit *et al.* 1959), in which one or more impulses appeared with a latency of 3–6 msec after the stimulus to the nerve. The number of impulses elicited was usually 2 or 3, rarely 6. The peak frequency of this discharge, measured as the reciprocal of the interval between the impulses, was 500–600 /sec as in stretch receptors (Hunt & Kuffler, 1959; Granit *et al.* 1959). The number of impulses in this 'early discharge' did not vary consistently with the tension applied; sometimes it fell with increase of initial tension. These impulses were abolished by curare or Flaxedil. It is possible they were ephaptic in origin, as has been postulated by Granit *et al.* (1959), but one cannot rule out mechanical stimulation with the evidence available at present. One observation that supports ephaptic excitation is that, although the response of one receptor to pressure was abolished by local mechanical injury, its fibre still yielded the 'early discharge'. The 'early discharge' must influence the subsequent response of the ending in view of the known effects of antidromic depression (Matthews, 1933; Paintal, 1959*b*), particularly if the train consists of several impulses. Perhaps these endings would have been excited during some phase of the twitch if the 'early discharge' were not there. Such a discharge was observed in two receptors in which an 'off-effect' type of response was seen in the form of 2–4 impulses after the active phase of contraction (Fig. 7*A*).

Since there was no resting discharge in these receptors it was considered worth while to see what would happen during a twitch if such a steady discharge could be created. Accordingly, the effect of a muscle twitch during application of local pressure was examined, and as expected there was no consistent response. Some receptors showed a pause during contraction, as in Fig. 6*B*, *C*, others a tendon-organ type of discharge (Fig. 6*D*, *E*, *F*). With constant pressure the discharge in one of these increased with increasing strength of contraction up to a point and then declined (Fig. 6*D*, *E*, *F*); this is of course quite unlike the discharge from tendon organs. In this receptor increase in the local pressure applied increased the number of impulses in the train.

Effect of tetanic contraction. The effect of tetanic contraction of muscle at 40–100 /sec was observed on 10 pressure receptors. Six of them were apparently unaffected by this procedure, although the muscle was

tetanized through its nerve at subthreshold strength for the afferent fibre so that its receptor was not depressed antidromically (Paintal, 1959*b*). The remaining 4 were stimulated to varying degrees. In two fibres tetanic stimulation at about 40/sec yielded a discharge of 1–2 impulses/sec in one fibre and about 6/sec in the other; this ceased soon after tetanus. In a third, stimulation at 90/sec produced a discharge of 10 impulses/sec which

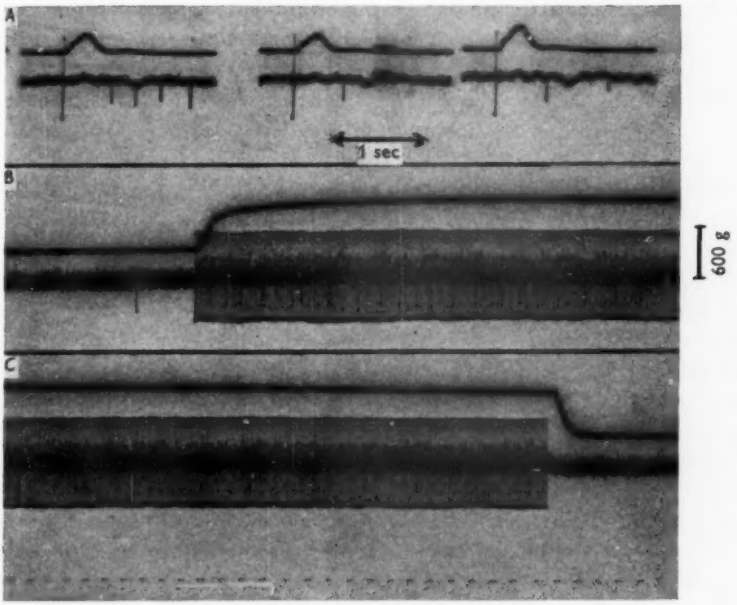

Fig. 7. Effect of muscular contraction on a lateral gastrocnemius-soleus pressure receptor. *A* shows variable responses to single muscle twitches in 3 successive sweeps. *B* and *C*, which are continuous records, show that tetanic muscular contraction stimulated the receptor. Nerve was stimulated at about 90/sec and the muscle was partially curarized. Lowest trace in *C*, time marker 1/10 sec. Conduction velocity of fibre was 11·4 m/sec.

started about 80 msec after the beginning of the tetanus (Fig. 7*B*). This discharge was not maintained throughout the period of repetitive stimulation and it was present during apparently constant tension, and in one trial while the tension was falling. Tetanus at about 40/sec yielded a similar response but the discharge frequency was lower; at 18/sec the receptor was not stimulated. In the fourth receptor a train of impulses at a frequency of about 10/sec appeared after the end of the tetanus and lasted for about 6 sec. The absence of discharge during tetanus in

this fibre was probably due to antidromic bombardment of the ending, as the stimulus used was suprathreshold for the afferent fibre. These results indicate that prolonged muscular contraction as in a tetanus can stimulate some pressure receptors, and that this excitatory action ceases with the end of the tetanus although in certain receptors it may outlast it.

Location of receptors. Only 10 pressure receptors have been located in the muscle; in the case of the others either no attempts were made to locate them or the attempts were unsuccessful. The method of location was quite simple. The region which yielded the maximum response to pressure was at first tentatively recognized as the location of the receptor. This region was then either destroyed or disconnected from the rest of the muscle, and if the response disappeared the tentative conclusion stood. If it did not, the procedure was repeated. To determine whether the receptors were in the soleus or lateral gastrocnemius the muscles were separated from one another and the procedure for location described above followed.

Three of the 10 receptors were located precisely in the musculo-tendinous region of the tendo Achillis; two were located in the fascia covering the flexor digitorum longus muscles. Of the remaining five receptors two were located in the belly of the lateral gastrocnemius muscle (one near its origin) and three in the belly of the soleus. These results indicate that many of the pressure receptors are probably located in the musculotendinous region of the muscle. This information may be of value for determining the central effects of these receptors.

Effect of asphyxia. As is well known, occlusion of the circulation or asphyxia has profound excitatory effects on muscle stretch receptors (Matthews, 1933; Paintal, 1959*b*). It was therefore of interest to see if the pressure receptors were similarly affected. Accordingly, the behaviour of pressure receptors was examined after stopping the respiratory pump (followed by cardiac standstill) in curarized animals. Out of 9 pressure receptors on which the effect of asphyxia was tried, only 1 was stimulated and this was connected to a Group I fibre with a conduction velocity of 91 m/sec. All the others, 7 of Group III and 1 of Group II, were not stimulated by asphyxia. The responses of some of the receptors to pressure lasted for as long as 1 hr after stopping the pump but in no case were their responses enhanced.

Effect of NaCl 6 % (*w*/*v*). Since the pressure receptors were not stimulated by any type of stimulus other than pressure, which had to be severe to stimulate some of them, the possibility that they were pain endings was considered, particularly since subjective experience indicates that squeezing a muscle produces pain—this local tenderness being marked after strenuous exercise. Further, Lewis has shown that severe muscular pain is produced by local injection of 6 % NaCl (Lewis, 1942). Accordingly,

the effect of locally injected 6% NaCl on some pressure receptors was noted. For this purpose observations were made exclusively on Group III pressure receptors.

After locating a receptor a No. 12 gauge needle was inserted into the site. This procedure itself evoked a train of impulses (Fig. 8*C*). This was apparently not a pressure effect of the type described above because the pressure exerted by the needle in some cases was much less than that required to stimulate the endings by pressure *per se*. Further, excitation

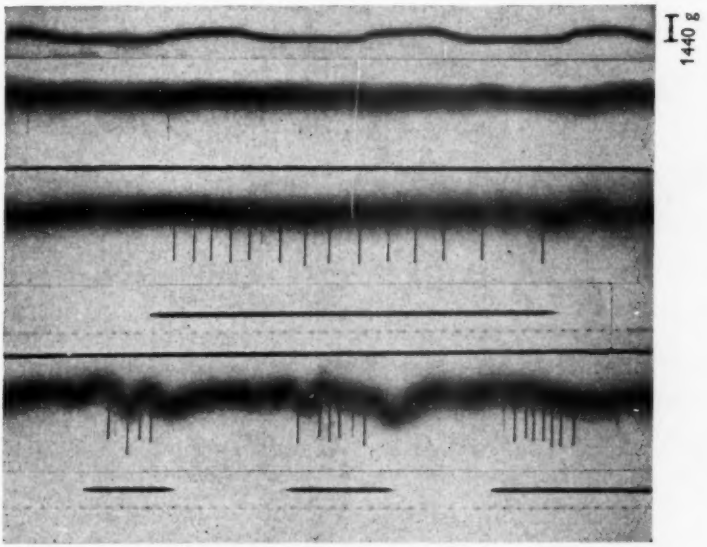

Fig. 8. Responses of a lateral gastrocnemius-soleus pressure receptor. *A* shows that stretching the muscle did not stimulate the receptor, whereas pressing the muscle at signal in *B* stimulated the ending. *C* shows that inward movement of a hypodermic needle inside the muscle at signals stimulated the receptor. Lowest trace in *B* and *C* time marker 1/10 sec. Conduction velocity of fibre was 17·1 m/sec.

occurred only after penetration of the muscle, at which time the pressure exerted by the needle was minimal. It appeared that this excitation was due to the movement or progress of the needle inside the muscle, because small displacements of the needle in and out of the muscle excited the receptor. In fact, in some receptors there was excitation when movement occurred in a particular direction, e.g. inward in the case of the receptor shown in Fig. 8*C*. The excitation by a needle was short-lasting, the discharge rarely exceeding 1 sec and being usually over within a fraction of a second. Occasionally this was followed by infrequent and irregularly occurring impulses with a frequency of less than 1/sec.

With the position of the needle unchanged about 0·5 ml. of 6 % NaCl was injected. In 8 out of 9 cases this yielded a discharge of impulses lasting from 2 to 5 min. Usually the discharge built up gradually as in Fig. 9 to reach peak frequency about 15–50 sec from start of stimulation. The latent period, reckoned from the beginning of injection to the beginning of stimulation, varied in different receptors from 1·3 to 23 sec. The longer latencies were probably due to the needle being at some distance from the endings and the interval therefore represents the time taken for the solutions to diffuse to the ending. The peak frequency was 3–30 impulses/sec.

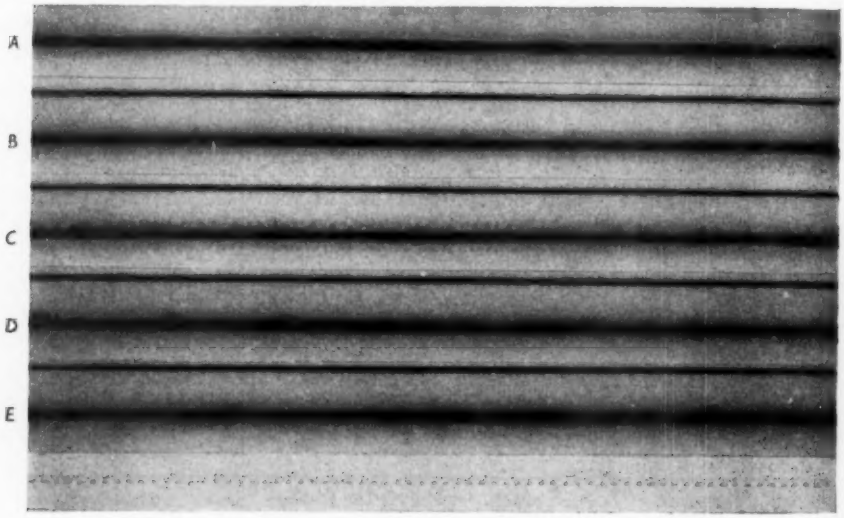

Fig. 9. Effect on a lateral gastrocnemius-soleus pressure receptor of locally injected 0·5 ml. 6 % NaCl. *A* is a record before injection of 6 % NaCl; *B–E* are records respectively at 50, 120, 175, and 260 sec after injecting 6 % NaCl. Lowest trace, time marker 1/10 sec. Conduction velocity of fibre was 14·1 m/sec.

After a period of continuous activity, as in Fig. 9*C*, cyclical discharges appeared in some receptors. This consisted of short trains of impulses lasting about 0·2–2 sec, separated by variable periods of silence (Fig. 9*D*, 10*C*). As time passed the duration of the trains diminished and the periods of silence increased (Fig. 9*D*, *E*).

After the excitation by NaCl had died down it was found that the response of the receptors to pressure was reduced. It was also found consistently that the response to a second injection of NaCl was less than that following the first. The response in Fig. 9 shows the effect of a second injection; the response to the first being much more marked.

The effect of NaCl 0·9 % (w/v) was tried on 4 receptors. This was injected in the same way before injecting 6 % NaCl. It had no excitatory action on 3 receptors that were markedly stimulated by 6 % NaCl. In one case the receptor was clearly stimulated, the discharge lasting for about 4 min (Fig. 10*B*). In this case 6 % NaCl stimulated the ending after a latency of only 1·3 sec (Fig. 10*D*), which is small when compared

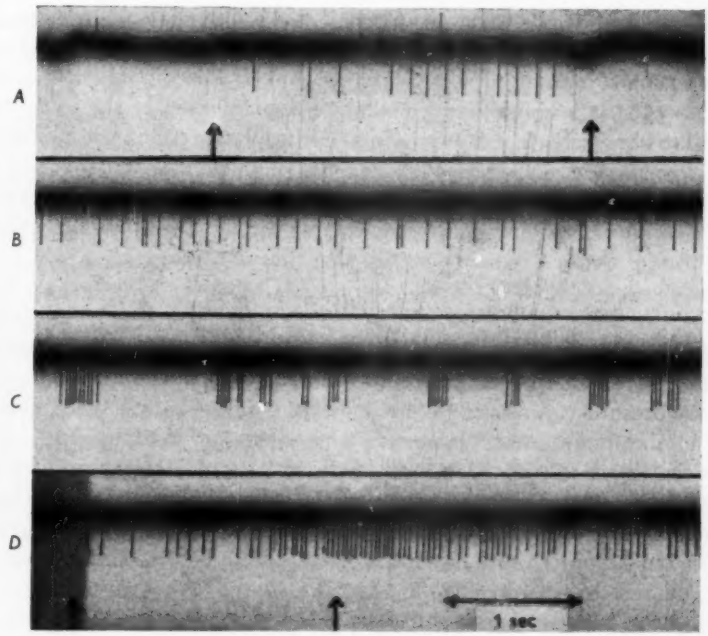

Fig. 10. Responses of a lateral gastrocnemius-soleus pressure receptor. *A* shows the response to local pressure applied to the muscle between arrows. *B* and *C* are records taken respectively at 7 and 30 sec after intramuscular injection of 0·5 ml. 0·9 % NaCl near location of receptor. *D* shows the marked and immediate stimulation of the receptor by injecting 6 % NaCl at the same place; the injection is indicated by arrows. Conduction velocity of fibre was 17·1 m/sec.

to the usual latency of 8–23 sec seen in the other receptors. It is conceivable that in this instance the hypodermic needle was closer to the ending than in the other cases.

The behaviour described above is the kind that would explain some of the subjective experiences of pain following intramuscular injection of 6 % NaCl in man (Lewis, 1942). Probably these pressure receptors are indeed responsible for the sensation of pain from 6 % NaCl, but the possibility that stretch receptors may also be involved has to be kept in mind.

The effect of 6% NaCl on stretch receptors was therefore tried. For this purpose the whole nerve of either medial gastrocnemius or lateral gastrocnemius-soleus was placed on the recording electrodes and 6% NaCl injected as before. This did not produce any visible change in the resting discharge although 0·5 ml., which is a very large dose for a cat's gastrocnemius compared to the 0·03 ml. used by Lewis (1942), was injected into several regions of all three muscles in a number of experiments. Occasionally a high-frequency discharge appeared in 1–4 fibres of unknown identity, especially if the solution was injected near the origin of the muscle. Larger doses (1·0 ml.) activated several fibres, but this discharge was only a fraction of that accompanying moderate ventriflexion of the foot. Since ventriflexion of the foot consistently produced a pronounced maintained discharge and since this procedure is quite painless, it is evident that pain following local infiltration of 6% NaCl is not mediated by stretch receptors. The receptors that qualify for this role so far are therefore the pressure receptors of Group III and possibly also those of Group II.

Receptors in tibialis anterior

The experiments on the receptors of tibialis anterior were undertaken after those on the lateral gastrocnemius and soleus had been completed and the results assessed. Figure 2 of Lloyd & Chang (1948) suggests that in the mixed lateral gastrocnemius-soleus nerve about 16% of the fibres belong to Group III (18% in lateral gastrocnemius and 14% in soleus). With this population of Group III fibres it was possible to isolate usually 1–2, rarely 3, Group III fibres in each experiment. This of course would depend on the skill of the experimenter and the techniques used by him. On the other hand, Fig. 3 of Lloyd & Chang (1948) shows that about 40% of the afferent fibres in tibialis anterior nerve belong to Group III. It was therefore expected that two to three times the number of Group III fibres isolated per experiment in the lateral gastrocnemius-soleus would be isolated in each experiment on the tibialis anterior.

Altogether four experiments were done on the left tibialis anterior and a total of 25 Group III fibres were isolated, an average of about 6 fibres per experiment (range 5–8 fibres/expt.). These were isolated without particular difficulty and they do not represent the maximum that could be isolated, because usually the experiment was terminated before the entire nerve could be examined. These results therefore confirm the expectations arising from the fibre calibre spectra of 'demotored' muscle nerves (Lloyd & Chang, 1948). More experiments on tibialis anterior were not done because the other results were similar to those obtained in the lateral gastrocnemius-soleus nerve.

The conduction velocities of the fibres isolated ranged from 5·2 to

22 m/sec, the maximum occurring in the 5–10 m/sec range. Relatively few fibres were isolated in the 20–25 m/sec group; this may have been due to some undetected bias for the smaller fibres in the sampling procedure. Assuming that the distance travelled by an impulse in 1 sec is equal to 6×10^6 times the fibre diameter (Hursh, 1939) the mean conduction velocity calculated for the Group III fibres in Fig. 3 of Lloyd & Chang (1948) would be about 15 m/sec. This is a little different from the mean of 11 m/sec found in the present experiments.

No impulses could be aroused in 13 of the 25 Group III fibres isolated by stimulating the muscle mechanically; 2, with conduction velocities of 18 and 22 m/sec, respectively, were connected to typical stretch receptors. Ten were connected to pressure receptors whose responses closely resembled those found in the lateral gastrocnemius and soleus. The range of threshold to pressure was also similar. Sometimes, presumably owing to altered conditions, the receptors which could be stimulated by pressure at one stage of the experiment could not be stimulated at all at another. These receptors represented the border-line behaviour between pressure receptors and inactive Group III afferent fibres. It is, therefore, possible that the 13 Group III fibres labelled as inactive could have been activated by pressure at another stage of the experiment. In this connexion the conditions of the experiment should be kept in mind, i.e. separation of muscles, etc. (see Methods).

An important observation, which indicates that there are certain minor differences in the behaviour of pressure receptors of different muscles, is that in 6 of the 10 receptors stretching the muscle rapidly to a tension of about 700 g yielded 1–10 impulses. This represents a greater excitation by stretch than that which occurred in the receptors of the lateral gastrocnemius and soleus muscles. Figure 11*B* shows a receptor which had the best response to stretch out of all the pressure receptors examined. However, the same receptor at an earlier stage of the experiment discharged only 1–3 impulses during stretching of the same degree.

The response to stretch shown in Fig. 11*B* is the kind which Matthews noted in his 'C' endings, most of which were presumably from peroneus longus muscle (Matthews, 1933). The pressure receptors resemble the 'C' endings further in that their responses during active contraction are variable and that some of them, especially those of lateral gastrocnemius and soleus, are attached to fascia.

Unlike those in the lateral gastrocnemius and soleus, a few receptors discharged impulses irregularly at a frequency of less than 1–2/sec under resting conditions. Such pressure receptors could always be stimulated mechanically.

Nine of the 10 pressure receptors were located within the muscle. Unlike

those in the lateral gastrocnemius and soleus the pressure receptors of tibialis anterior were concentrated near the entry of the nerve into the muscle. Two were located about 5 mm central to the entry of the nerve, 4 were located within 1 cm distal to the entry of the nerve, and the remaining 3 within 3 cm distal to the entry of the nerve. The total length of the muscular portion of the muscle is about 8 cm and the nerve enters it about 1·5 cm from its origin.

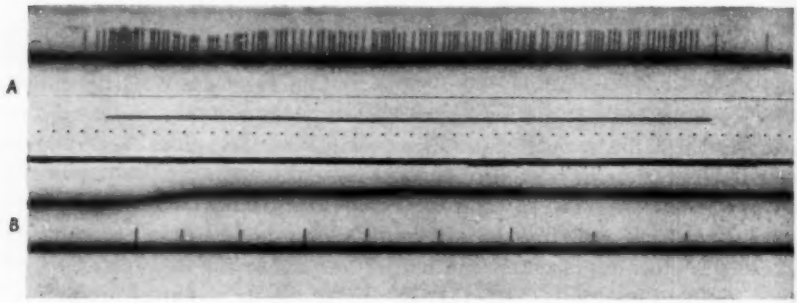

Fig. 11. Responses of a tibialis anterior pressure receptor. *A* shows the response to gentle local pressure. *B* shows the response of the same receptor to stretching the muscle from zero initial tension to 780 g. Lowest trace in *A*, time marker 1/10 sec. Conduction velocity of fibre was 11·1 m/sec.

Electrical threshold of Group III fibres

This was carefully determined in 19 fibres. The threshold was evaluated relative to the threshold of motor fibres; threshold of Group I afferent fibres could not be used as a reference owing to the conditions of the experiments. The threshold ranged from 7 to 44 times the threshold for motor fibres; the average was 24. The duration of the stimulus was kept constant at 0·1 msec. The results indicate that with a stimulus about 20 times threshold for motor fibres about half the total number of Group III fibres would be stimulated.

DISCUSSION

The main conclusion emerging from the experiments is that the majority of Group III fibres in muscles are connected to pressure receptors. Some stretch receptors are also connected to Group III fibres but the number of these is very small. Further, since the results obtained in the lateral gastrocnemius and soleus are similar to those obtained in the tibialis anterior it may be assumed that they apply to other muscles as well.

A great deal of histological study has been devoted to the sensory endings in muscles (for references see Barker, 1948), but there have been

no reports concerning presence of specific structures other than muscle spindles, Golgi tendon organs, and occasional Pacinian corpuscles. There is, therefore, a possibility that some of the Group III fibres merely terminate in naked endings. This is the sort of termination attributed to pain fibres. (Wolff & Wolf, 1951) Recently Barker (1959) has reported a fair number of Pacinian corpuscles in the rectus femoris. It would be interesting to know the size of the nerve fibres supplying these corpuscles.

The Group III fibres probably produce several types of central effects. First, there is a possibility that they mediate the sensation of muscle pain, although there is no actual proof to show that this is so. However, there is some evidence to support this view if it is assumed that the Group III pressure receptors in man respond in the same way as those in cats. First, many of the pressure receptors are stimulated only by strong pressure, e.g. 1–2 kg/cm^2, just that pressure required to produce the pain sensation in man. Secondly, preliminary experiments indicate that the pain produced by squeezing a muscle (e.g. calf muscles) is conducted fast enough for it to be mediated by myelinated afferent fibres, which the Group III are. Finally, the pressure receptors are stimulated markedly by locally injected 6 % NaCl, which produces severe pain in man (Lewis, 1942). The quantities injected into cats (0·5 ml.) might seem large when compared to the quantities required to produce pain in man (0·02–0·03 ml.; Lewis, 1942), but it must be remembered that in the present investigation 6 % NaCl was used to stimulate single pressure receptors which could only be localized in two dimensions but not the third, namely depth. In order to stimulate the endings, therefore, the seemingly large quantity of 0·5 ml. was injected so that it could diffuse to particular pressure receptors in effective concentrations. It is certain that if the solution could be introduced precisely at the receptors only very small amounts would be needed. The above evidence suggests that the Group III pressure receptors may be responsible for some aspects of muscle pain, in which case they could be appropriately referred to as 'pressure–pain' receptors. However, the evidence is still only circumstantial and further experiments on man will be needed to establish the point. In this connexion the role of non-myelinated fibres must be kept in mind because there are endings connected to non-myelinated fibres which are stimulated by strong pressure but not by external stretch (unpublished observations).

According to Lewis the pain accompanying muscle activity during ischaemia is of the same quality and presumably arises from the same source as that produced by squeezing the muscle (Lewis, 1942, p. 41). The results of this investigation do not fit into this scheme, because the pressure receptors are not stimulated by asphyxia and some not even by tetanic contraction during asphyxia. It is possible that there is more than

one pain-conducting mechanism and the fact that there are several types of non-myelinated afferent fibres in muscle (unpublished observations) supports this possibility. At any rate it can be concluded that Group III pressure receptors are probably not responsible for the pain of muscular ischaemia.

Since Group III fibres of muscles facilitate flexor reflexes (Lloyd, 1943; Brock, Eccles & Rall, 1951; Eccles & Lundberg, 1959), it may be concluded that the Group III (and perhaps Group II) pressure receptors of different muscles produce the flexor reflex, especially since the majority of Group III fibres are connected to pressure receptors. This is consistent with their probably nociceptive function outlined above. Recently Eccles & Lundberg (1959) have shown that the interneurones concerned with this pathway are inhibited by higher centres in the brain stem, because the flexor reflex of Group II and Group III fibres, which is insignificant or absent in the decerebrate cat, comes into prominence on dividing the cord in the upper cervical regions. However, the reflex effects of Group III fibres on muscles of the opposite limb (Perl, 1958) suggest that their reflex actions must be complex.

A third possibility is that the pressure receptors are responsible for the increase in ventilation during muscular activity. From the experiments of Iaria, Jalar & Kao (1959) it would appear that the stretch receptors of muscle are not responsible for this increase in minute ventilation on stimulation of a muscle nerve. As shown in this investigation some pressure receptors are stimulated by muscular contraction and if the stimulus strength is high enough the Group III fibres themselves may be stimulated. The possibility that their activity increases ventilation has therefore to be kept in mind. Experiments to test this possibility are in progress.

Finally, the observations of Hunt & Paintal (1958) on the effects of Group III fibres on fusimotor neurones (gamma fibres) must be kept in mind.

The Group II band of afferent fibres can no longer be regarded as a homogeneous group of stretch fibres, owing to the presence of a fair number of fibres connected to pressure receptors (Fig. 4). For the present, the central effects of these receptors may be considered to be the same as those of the Group III ones.

SUMMARY

1. By recording impulses in fibres of known conduction velocity it has been established that the majority of Group III sensory fibres in lateral gastrocnemius, soleus and tibialis anterior muscles terminate in pressure receptors, very few in stretch receptors; in some no impulses could be aroused by mechanical stimuli.

2. The threshold of pressure receptors varied considerably. Most of them adapted rapidly to the stimulus. With a few exceptions they were not stimulated by stretching the muscle. Some yielded a variable number of impulses during a muscle twitch. Tetanic contraction of muscle produced a low-frequency discharge in about half the pressure receptors.

3. Most of the pressure receptors of tibialis anterior were located near the point of entry of the nerve into the muscle. In the lateral gastrocnemius and soleus more were located near the musculo-tendinous region; others in the belly of the muscles.

4. The pressure receptors of lateral gastrocnemius and soleus were connected to afferent fibres with conduction velocities ranging from 6 to 91 m/sec. Most of them were connected to Group III fibres (maximum in the 10–15 m/sec range), some to Group II and very few to Group I fibres. There appear to be more Group III pressure receptors in tibialis anterior than in the lateral gastrocnemius and soleus.

5. Some Group III pressure receptors were stimulated by introducing a hypodermic needle into the muscle at the localized site. Nearly all were stimulated strongly by injecting 0·5 ml. NaCl 6% (w/v) at this site; the duration of stimulation lasted about 2–5 min.

6. It is suggested that the Group III pressure receptors may mediate the sensation of muscle pain produced by squeezing the muscle or injecting 6% NaCl locally (Lewis, 1942); Groups I and II stretch receptors are not responsible for this pain. The Group III pressure receptors are probably not responsible for the pain of muscle ischaemia, because none of them were stimulated following asphyxia and cardiac standstill, some not even by tetanic contraction of muscle during this period.

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SOME OBSERVATIONS ON FOETAL AND NEW-BORN RHESUS MONKEYS

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(*Received* 4 *January* 1960)

The object of the experiments to be described was to provide physiological data on the condition of the foetal monkey *in utero* and after delivery, with particular regard to the cardiovascular and respiratory systems and the ability to withstand acute oxygen lack. A preliminary account of part of this work has been given elsewhere (Dawes, Jacobson, Mott & Shelley, 1960).

METHODS

Observations were made upon twelve pregnant rhesus monkeys and their foetuses (of 115–158 days gestation age) and on six monkeys from birth up to 8–12 days of age. Additional information was also obtained from one monkey delivered vaginally, partly asphyxiated, at 117 days gestation, one 15-day-old monkey and two aged 1–2 years.

Foetal monkeys. The pregnant females were anaesthetized with pentobarbitone (the initial dose required was usually 20–25 mg/kg), and an endotracheal tube was passed and fixed in position. The monkey was placed on a warm table, and covered with towels to reduce heat loss. Rectal temperature was recorded at intervals during the experiment. The abdomen was opened by a paramedian incision. In the older foetuses (140 days gestation or more) the head was deep in the maternal pelvis. The extent of the placenta was determined and a foetal leg was withdrawn through a small incision in the uterus, so that a catheter could be tied into the femoral artery. In four experiments an arm was also withdrawn through a separate incision and a catheter was tied into a brachial artery. In the younger foetuses (126 days gestation or less) the head was not yet fixed in the pelvis. The foetus was turned into a convenient position *in utero*, a small uterine incision was made over the neck and a catheter was placed in the carotid artery without removing the foetus from the uterus. This manoeuvre was performed because of the difficulty expected in cannulating the femoral artery in the smaller foetuses.

Blood samples (0·7–1·0 ml.) were withdrawn into a syringe, the dead space of which was filled with a solution containing 0·4 % heparin and 6 % (w/v) NaF. Blood pressure and intrauterine pressure (from a saline-filled balloon introduced into the amniotic sac) were recorded with a mercury manometer. At the end of each experiment samples of heart, lung, liver, muscle and placenta were removed as rapidly as possible, usually in that order.

In the first three foetal experiments the mother was killed so that samples of various tissues could be obtained, and the skin was removed for the measurement of surface area. The nine subsequent experiments were performed with sterile precautions; the uterus and

the abdomen were closed after delivery of the foetus and placenta and the mothers made an uninterrupted recovery.

New-born monkeys. The mothers were kept in an animal house at a temperature of 23–26° C. The baby was removed as soon as practicable after natural birth and placed in a child's incubator, maintained at 30° C and humidity 60–65 % and ventilated with room air. As birth usually took place at night, there was sometimes a delay of a few hours before the baby was placed in the incubator. During the first 24 hr after birth the monkey was not fed unless it showed signs of hunger, when it was given 10 % glucose every 2 hr. For the next 3 days it was fed on milk by bottle every 2 hr, and thereafter less frequently. After 8–12 days the monkeys were anaesthetized with pentobarbitone (initial dose 15–26 mg/kg) and an acute experiment was performed, after which samples of various tissues were removed.

Oxygen consumption

Unanaesthetized new-born monkeys were placed, according to size, in one of two cylindrical glass vessels 11·5 cm high and 11·5 cm internal diameter, or 13·0 cm high and 13·5 cm internal diameter. They were given a small piece of rag to clutch and appeared more comfortable when lying on a soft gauze pad. The top of the vessel was sealed by a cover pierced with three holes, one of which was blocked by a thermometer used to record the environmental temperature. The vessel was attached by the other two holes to a closed circuit, consisting of 1–3 aquarium aerators, a soda-lime container, a rotameter and a small volume recorder. The aerators were adjusted to give a total flow of 1·5–2·0 l./min. The combined volumes of either glass vessel and the external circuit were 1·3 and 2·0 l. before the monkey was placed in them. The float of the volume recorder was $6{\cdot}5 \times 5{\cdot}0$ cm² in area and this, with a pointer 36·5 cm long, gave a sensitivity of 8·8 ml./inch (2·5 cm). The rate of O_2 consumption was measured by recording, with a stopwatch, the time taken for the pointer to fall 1 in., during which time the O_2 content of the circuit would fall not more than 1 %. Sufficient O_2 was then added to the system to return the pointer of the volume recorder to its initial level, and the procedure was repeated. At the beginning of each experiment O_2 was added to raise the initial O_2 content to 23–29 %. The glass vessel and parts of the external circuit were immersed in a large water-bath, the temperature of which was controlled to within $\pm 0{\cdot}15$° C by adding warm or cold water or ice by hand. The temperature of the volume recorder was noted at intervals, and all observations were corrected to and are expressed as at s.t.p. in dry air. After the monkey had been introduced into the closed circuit, and the temperatures of the bath and within the glass vessel had become steady, consecutive measurements of the time required to consume 8·8 ml. O_2 were made and plotted against time. The mean of ten such measurements, covering a period of 15–20 min, was taken as a single estimate of O_2 consumption, and such estimates were usually repeated two or more times at each environmental temperature.

The O_2 content of the closed system was measured by withdrawing a 10 ml. sample at a time when the volume recorder pointer had reached the mid point of its excursion. This sample was analysed by the method of Gibson, Greenfield & Thompson (1953). The O_2 content of the system was reduced by refilling with air, instead of with O_2, at the end of each excursion of the volume recorder, until the desired fall in O_2 content had been attained. The O_2 content of the system was raised by opening it for a short while to the atmosphere and by running in O_2 from a cylinder.

Anaesthetized new-born monkeys. The O_2 consumption was measured by attaching a tracheal cannula in series with the system as described, including in it the glass vessel containing 500 ml. water. The total volume of the system was then similar to that used when an unanaesthetized monkey was placed within the glass vessel.

Anaesthetized adult female monkeys. The O_2 consumption was measured by attaching an endotracheal tube in series with the system, including in it the empty glass vessel, but with a larger volume recorder (float 8×10 cm² area) and a larger gas flow (3·5 l./min).

The surface area of new-born and adult monkeys was measured by removing the skin post-mortem, stretching it gently upon a sheet of cardboard of known weight per unit area, tracing the outline, and weighing the cardboard within the area of the tracing.

Biochemical and histological methods

Blood samples were analysed for O_2 content by the micro-method of Roughton & Scholander (1943). The O_2 content of fully saturated samples was also estimated, so that the percentage O_2 saturation could be calculated. The relative haemoglobin content of each blood sample was estimated by using a Beckman Model B spectrophotometer to measure the optical density at 540 mμ of 0·03 ml. blood in 7·5 ml. dilute ammonia (ammonia (sp.gr. 0·9) 0·4 ml./100 ml.). These measurements were used to check the changes in oxygen-carrying capacity of the blood from one sample to another during the course of an experiment.

Other biochemical and histological methods were those used by Dawes, Mott & Shelley (1959), with the exception that blood glucose and blood lactate determinations were made on the same extract, prepared by sodium hydroxide–zinc sulphate protein precipitation.

RESULTS

Observations on foetal monkeys

The upper part of Table 1 shows the results of analysis of the first blood samples withdrawn from eleven foetal monkeys, that is, samples taken as soon as a catheter had been introduced into a carotid artery

TABLE 1. Analysis of arterial blood samples withdrawn from foetal and new-born monkeys

With the exception of the three youngest foetal monkeys* (111, 114, 113), in which they were taken from a carotid artery, all samples were taken from a femoral artery. The lactate figures in parentheses indicate values observed an hour after the initial samples were withdrawn.

Foetus No.	Gestation age (days)	Age from birth (days)	Weight (g)	Arterial O_2 saturation (%)	Blood lactate (mg/100 ml.)	Blood glucose (mg/100 ml.)
111 ♀	115	—	200	60*	10 (14)	24
114 ♂	116	—	226	77*	8	29
113 ♂	126	—	264	55*	17	26
103 ♂	140	—	356	57	17 (13)	44
106 ♂	140	—	340	63	13 (11)	36
112 ♂	141	—	360	63	6 (10)	27
105 ♂	142	—	347	58	7 (8)	20
104 ♀	146	—	394	54	16 (10)	34
100 ♂	148	—	387	56	26 (14)	36
109 ♂	153	—	401	62	7 (14)	20
98 ♂	158	—	407	51	16	26
					Mean	29
110 ♀	145	—	528	15	68	32
New-born No.						
101 ♀	162	12	395	—	7 (7)	46
96 ♀	169	10	405	—	11 (14)	65
108 ♂	172	10	521	93	7	50
102 ♀	173	8	531	—	13 (14)	47
107 ♂	174	9	561	94	9	48
94 ♀	177	9	517	—	17 (12)	37
					Mean	49

(in the three youngest foetuses) or a femoral artery (in the remainder). The figures (excluding those of No. 110 which will be considered separately) show that the O_2 content of the arterial blood in the foetal monkeys under pentobarbitone anaesthesia was high. The mean saturation in the femoral artery was 58%. There was no obvious variation with increasing gestational age, but the femoral arterial O_2 saturation appeared to increase with the order in which the experiments were performed (as indicated by the numerals 98–112 in the left-hand column).

In four foetuses after an initial femoral arterial sample had been

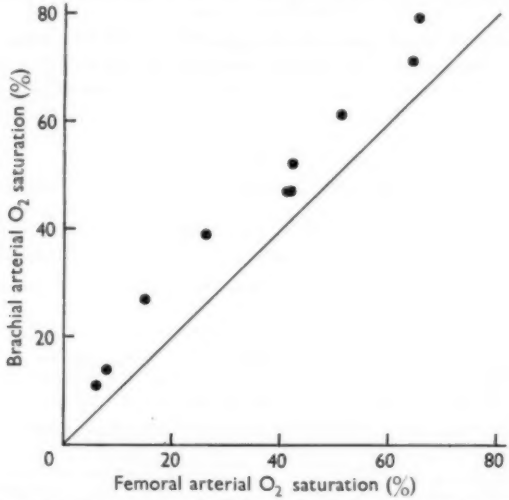


Fig. 1. Blood samples taken simultaneously from a brachial and a femoral artery in foetal monkeys. The O_2 saturation of blood from a brachial artery was always higher than that from a femoral artery.

obtained (from a leg delivered through a small uterine incision) an arm was delivered through a separate incision and the brachial artery was catheterized. In a fifth foetus simultaneous blood samples were taken from a femoral and brachial artery after delivery from the uterus; these provided the two lowest measurements in Fig. 1. As Fig. 1 shows, the O_2 saturation of brachial arterial samples was consistently higher than that of femoral arterial samples withdrawn simultaneously, irrespective of the absolute level of the O_2 saturation. The blood from the brachial artery had an O_2 saturation which was on the average 9% higher than that of femoral arterial blood. The highest values observed in the brachial or carotid artery were 79 and 77%.

Figure 2 suggests that there was little change in the oxygen-carrying capacity of the blood during the last third of gestation or, indeed, after

birth. When individual values were compared, however, the oxygen-carrying capacity of foetuses of 140 days gestation or more was always greater than that of their mothers, by a mean of 15%.

The estimates of blood lactate in Table 1 are accompanied by figures in parentheses which indicate the values observed 1 hr after the first blood samples had been withdrawn. Lactate is a slowly diffusible substance, and is only slowly removed from the blood stream after a period of muscular activity or a hypoxic episode. It is therefore reasonable to compare the lowest foetal values observed with the range of values found in the

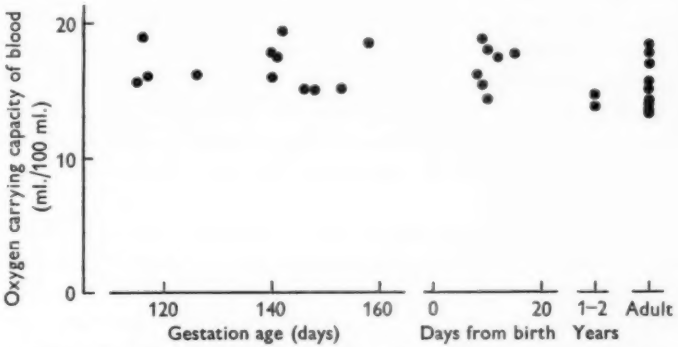


Fig. 2. The oxygen-carrying capacity of the blood plotted against age for foetal, new-born, adolescent and adult monkeys.

new-born monkey. It will be seen that there is no significant difference between these figures, an observation which suggests that, at least under pentobarbitone anaesthesia, the monkey foetus is not short of oxygen, and makes it doubtful that anaerobic glycolysis is a normal accompaniment of intra-uterine life. The mean blood glucose concentration in foetal monkeys was 29 mg/100 ml. This is significantly less than that observed in new-born monkeys (49 mg/100 ml. in Table 1). There was no consistent difference between the lactate and glucose concentrations of simultaneous femoral and brachial arterial blood samples.

When the uterus was first exposed within the abdominal cavity, contractions were infrequent and feeble. When the uterus was incised and the amnion opened, some fluid escaped and contractions became more regular and more powerful over the next 1–2 hr. During contractions the intra-uterine pressure rose by 5–15 mm Hg in monkeys which were not more than 126 days pregnant, and by up to 35 mm Hg later in pregnancy. The foetal heart rate, which was normally 150–170 beats/min, sometimes rose by as much as 20 beats/min at the peak of a contraction. In three foetal monkeys of 140–153 days' gestation a catheter was introduced into a femoral

artery and the leg, together with a saline-filled balloon, was returned to the uterus, which was then sewn up around the catheters and replaced in the abdomen. Blood samples taken from the femoral artery at or just after the peak of a uterine contraction consistently contained less O_2, by from 1·5 to 9% O_2 saturation, than samples withdrawn during a quiescent period between contractions.

During the course of the 1–2 hr following the first withdrawal of a leg from the uterus and the insertion of a femoral arterial catheter, the arterial O_2 saturation showed little change in some foetuses, whereas in others it fell considerably. In four foetuses in which it did not fall below 40% there was no significant change in blood glucose or lactate concentration. In foetuses in which the arterial O_2 saturation fell to lower values, either as a result of deterioration of the mother or foetus during a long experiment, or as a result of delivery, the blood glucose and lactate began to rise. There was no fall in heart rate during gradually progressive hypoxia until the femoral arterial O_2 saturation had been below 10% for some time.

In order to deliver the foetus the uterine incision, through which a limb had been withdrawn, had to be lengthened by 3–4 times in order to admit the foetal head. As soon as the foetus was removed, the uterus contracted strongly. The placenta or placentas became easily visible as they presented in the uterine incision. The colour of the blood in the umbilical vessels rapidly became darker and blood samples taken from a femoral artery as soon as practicable after delivery showed that the O_2 saturation had fallen to 8–32%. At this time the placenta had not separated; the foetuses sometimes made an occasional gasp, but were prevented from inhaling air by a saline-filled bag placed over the head. It was concluded that, because of the very strong contraction of the uterus and of the arrangement of the placenta, it was not possible to keep the monkey foetus in good physiological condition after delivery from the uterus.

In Fig. 3 are shown the weights of the twelve foetuses and six new-born monkeys plotted against gestation age, and also the weights of the placentas, including the membranes. It is evident that over the last 50 days of gestation the weight of the foetus approximately doubled, but that of the placenta and membranes showed little change. The weight of the membranes accounted for 12–25% of the combined weight of placenta and membranes. The weights of the uterus in three monkeys which were 140–158 days pregnant were 85–121 g, i.e. much the same as the weight of the placentas. In five instances there was a single or conjoined placenta and in seven others the placenta was double, and the umbilical vessels ran first to one placenta and thence to a second. The weight of the second placenta was from 35 to 59% of the total. The number and arrangement of the vessels

joining the two placentas was very variable, but had one of these vessels been occluded or catheterized there would have been a risk of depriving the foetus of a substantial proportion of its placenta.

The observations upon one foetal monkey were so different from those on any other that it has hitherto been excluded from consideration. This foetus (No. 110) was 145 days gestation age and this agreed with its sparse hair and general appearance, though it weighed 528 g, an exceptionally high value (Fig. 3). Meconium was present on opening the amnion, the

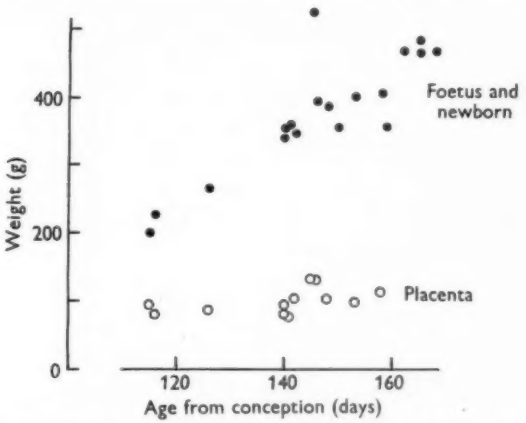


Fig. 3. The weights of twelve foetal monkeys (●) and of their placentas with membranes (○) plotted against conceptual age, together with the weights of six new-born monkeys (●).

femoral arterial O_2 saturation was only 15%, and the blood lactate was 68 mg/100 ml. The leg was returned to the uterus. During the next 1½ hr the foetus deteriorated even further. The arterial O_2 saturation fell to 2–8%, the blood lactate rose gradually to 121 mg/100 ml., and the blood glucose from 32 to 65 mg/100 ml. The pulse rate was low, about 130/min, rising to about 160/min during uterine contractions of up to 35 mm Hg. The blood pressure was low, 33 mm Hg, falling to 18 mm Hg on delivery. The placenta of this infant was large (134 g with membranes); it was the only placenta to show infarcts, of which there were five, the largest being 1 cm^2 in area. It was concluded that this monkey was grossly anoxic throughout the period of observation.

The oxygen consumption of new-born and adult monkeys

Without anaesthesia. Observations were made on six monkeys, unanaesthetized, from birth until 8–12 days of age. Four of them (162–168 days gestation age) were above the average weight at birth, while the

other two (150 and 159 days gestation age) were below the average weight (Fig. 4). The monkeys were kept at an environmental temperature of about 30° C and were fed with a milk mixture 2–3-hourly after the first 24 hr (see Methods). There was usually a small loss of weight after birth, which was made up within a few days. The least mature infant, born at 150 days gestation, was rather limp and did not suck well for the first 2 days, and she was also the slowest to regain her birth weight (101, ● Fig. 4). There is little doubt that the rectal temperature fell to a low value

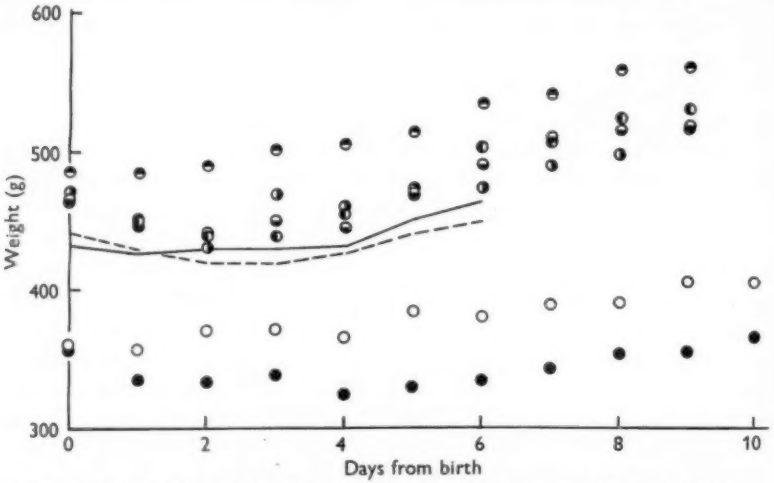


Fig. 4. The weights of the six monkeys, whose O_2 consumption was studied, plotted against age from birth. For comparison the continuous line indicates the mean weights of nine monkeys born by normal vaginal delivery at 151 days gestation or more, and the interrupted line indicates the mean weights of seventeen monkeys delivered by Caesarean section at 157–161 days gestation, over the first 7 days of life.

very soon after birth. In one monkey 16 min after a normal vaginal delivery it was 33·2° C; 2 hr later it had risen to 37·2° C. In the other monkeys it was 35·7–37° C when first measured 1–12 hr after delivery. In all except one the rectal temperature rose to 38° C or above by the fourth day from birth.

New-born monkeys shivered visibly when they were placed in a cold draught. Five were placed in a cool environment within 6–18 hr of birth, and in all of them the rate of O_2 consumption, which was 9·3–12·5 ml./kg. min at an environmental temperature of 35° C, increased very greatly on cooling. In three monkeys at 22–24° C it rose to 22–27 ml./kg. min. An example of experiments on one monkey is shown in Fig. 5, which also illustrates the fact that feeding has little immediate influence on the result.

The neutral zone, at which O_2 consumption was minimal, was about $35 \pm 1{\cdot}5°$ C over the first 8–12 days of life in all six monkeys. Above this environmental temperature they became restless and O_2 consumption increased (Fig. 5).

The rate of O_2 consumption at an environmental temperature of 35° C rose in the days following birth (Fig. 6). In all but the least mature monkey (101, ●) it rose within the first 24 hr; in No. 101 it rose after 48 hr. Thereafter there was a progressive increase in all monkeys, which appeared to reach a plateau between the sixth and twelfth days from

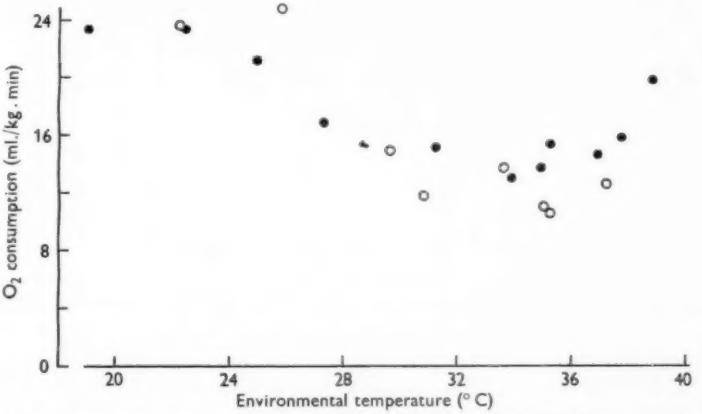


Fig. 5. Monkey No. 94. The rate of O_2 consumption/kg body wt. plotted against the environmental temperature 5–10 hr from birth (○) and 29–35 hr from birth (●). This monkey weighed 470 g at birth, 450 g the next day, and was fed 2–3 hourly from 24 hr onwards.

birth. This process was not followed further because it was considered desirable, for other reasons, to perform an acute experiment upon the monkeys when 8–12 days old. With increasing age not only did the rate of O_2 consumption at 35° C rise, but also that at lower temperatures, even below the temperature of 30° C at which the monkeys were kept in their incubator (Fig. 7).

Experiments were also carried out to test the performance of the apparatus for measuring O_2 consumption, and to see the behaviour of a new-born monkey during this procedure. Observations were made on each monkey for many hours during the first day after birth, and almost every day thereafter. The monkeys became quite familiar with the apparatus and usually lay down and went to sleep within a short while of being placed in the glass vessel. The respiratory rate did not change and gas analysis showed no evidence of CO_2 accumulation or of leaks. The lowest line on

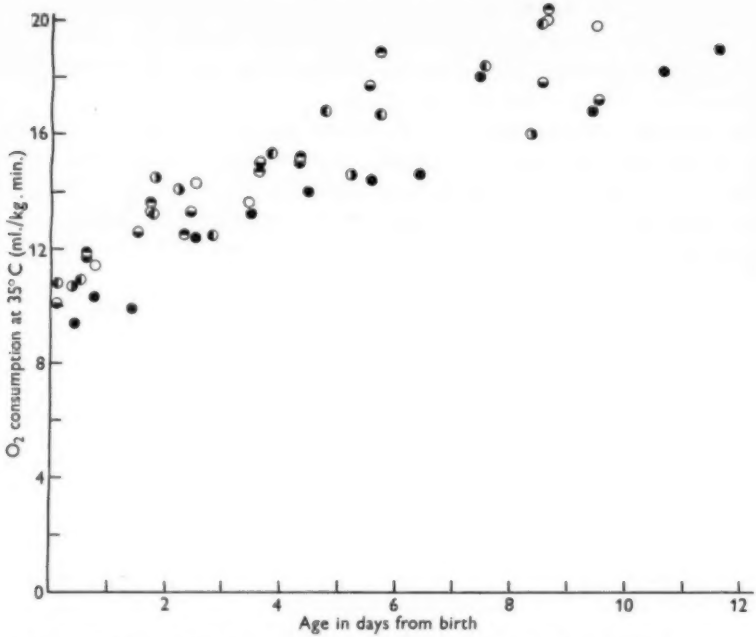


Fig. 6. The rate of O_2 consumption/kg body weight at an environmental temperature of 35° C plotted against age from birth for six monkeys. The symbols used for each monkey correspond to those used in Fig. 4.

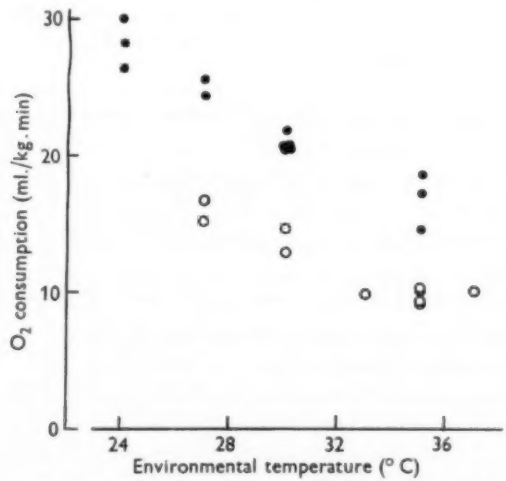


Fig. 7. Monkey No. 101. The rate of O_2 consumption/kg body wt. plotted against the environmental temperature during the first (○), and the seventh and eighth (●), days of life.

Fig. 8 (– – – –) shows the steadiness of the rate of O_2 consumption of a new-born monkey at 35° C over a period of 2 hr. They only rarely became restless and the increase in oxygen consumption observed during such activity was usually less than 2·5 ml./kg.min. When a monkey was subjected to a low environmental temperature, 22–26° C, the rate of O_2 consumption at once increased. In some instances as in that shown in the

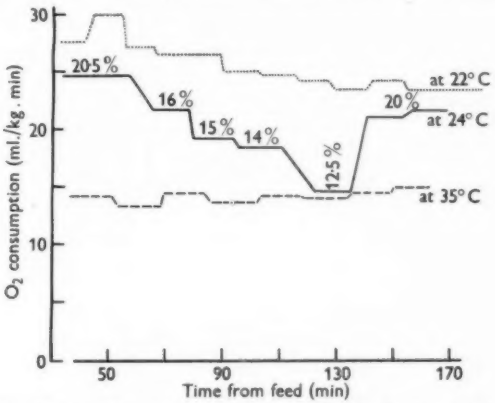


Fig. 8. Monkey No. 94. The rate of O_2 consumption/kg body wt. plotted against time from the last feed, at environmental temperatures of 35° C (– – – –), 22° C (.....) and 24° C (———). During the experiment at 24° C the O_2 content of the inspired gas was progressively reduced from 20·5 to 12·5 %, and then raised to 20 %, as indicated.

upper line (. . . .) of Fig. 8 at 22° C, there was a gradual small decline in O_2 consumption during the first 1½ hr of exposure; when this monkey was removed from the cold environment its rectal temperature had fallen from 38 to 30·7° C. In other experiments on monkeys exposed to 24–26° C the decline in O_2 consumption was less, and the rectal temperature at the end of the experiment was 34–36·7° C.

The effect of a decrease in the O_2 content of the inspired air was also examined. In four monkeys at an environmental temperature of 35° C a decrease to 10–14 % caused no significant change in O_2 consumption. However, in a cool environment, in which O_2 consumption was raised, lowering the O_2 content caused a fall in O_2 consumption. Figure 8 (——) shows the progressive fall in O_2 consumption of a 6-day-old monkey at 24° C as the O_2 content of the inspired air was lowered below 16 %. Under these experimental conditions the O_2 consumption fell towards the level observed at an environmental temperature of 35° C. Figure 9 shows the change in O_2 consumption of all six infant monkeys at an environmental temperature of 22–26° C, when the O_2 content of the inspired air was reduced. A temperature of 22–26° C was chosen because this was the

temperature range at which O_2 consumption was greatest (Fig. 5). Figure 9 demonstrates that the O_2 content of the inspired air usually had to be reduced below 15% before there was a significant fall in O_2 consumption under these conditions. The symbols used in this figure indicate the same monkeys as in Figs. 4 and 6; there did not appear to be any difference between the behaviour of the smaller (○, ●) and the larger monkeys (◓ ◐ ◒ ◑).

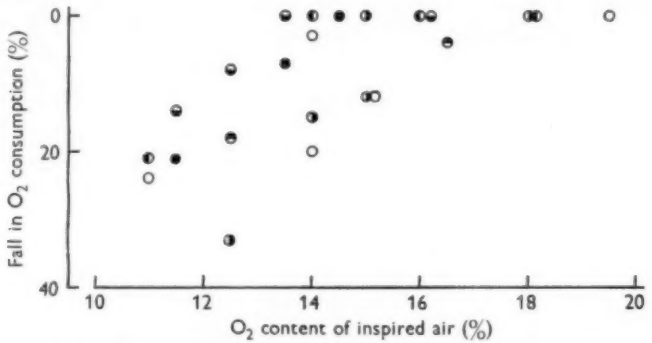


Fig. 9. The rate of O_2 consumption of six infant monkeys was measured at an environmental temperature of 22–26° C. The figure illustrates the decrease in O_2 consumption when the O_2 content of the inspired gas was reduced. The symbols used for each monkey correspond to those used in Figs. 4 and 6.

Under anaesthesia. When they were 8–12 days old, the monkeys were anaesthetized with pentobarbitone, and O_2 consumption was measured at the neutral temperature. Similar observations were made on eight anaesthetized pregnant female monkeys. These observations are summarized in Table 2, which also shows the surface area of the infant monkeys and of three pregnant females. The O_2 consumption of the anaesthetized infant monkeys (mean 14·8 ml./kg.min) was consistently less than observations made without anaesthesia the same or the previous day (mean 18·7 ml./kg.min). The mean O_2 consumption of the anaesthetized adult females was 6·1 ml./kg.min. The surface area per kilogram body weight of the infant monkeys was more than double that of the adult pregnant females, and their rate of O_2 consumption per kilogram body weight was greater by an almost identical ratio. Thus, under these particular experimental conditions the rate of O_2 consumption per unit surface area was similar in both 8–12-day-old monkeys and adult pregnant females.

Cardiovascular and respiratory reflexes in infant monkeys

Various observations were made on the six anaesthetized infant monkeys after their O_2 consumption had been measured. The blood pressure

was recorded from a brachial or femoral artery. Figure 10, which includes values obtained from foetal monkeys, shows that the blood pressure rose gradually during the last third of gestation. The measurements on the infant monkeys have been plotted against their conceptual age rather than age from birth, because the monkey (No. 101) which

TABLE 2. The oxygen consumption of anaesthetized infant (8–12-day-old) and adult female monkeys in the neutral environment

New-born No.	Weight (kg)	Surface area		Oxygen consumption		
		(m^2)	(m^2/kg)	(ml./min)	(ml./kg.min)	(ml./m^2.min)
101	0·395	0·048	0·121	5·62	14·2	117
96	0·405	0·047	0·116	6·92	17·1	147
94	0·517	0·050	0·097	7·03	13·6	141
108	0·521	0·060	0·115	7·68	14·7	128
102	0·531	0·058	0·109	8·22	15·5	142
107	0·561	0·064	0·114	7·72	13·8	121
		Mean	0·122		14·8	
Adult						
Q320	4·68	—	—	32·2	6·9	—
Q314	5·56	0·275	0·049	—	—	—
Q185	5·90	0·348	0·054	50·7	8·6	146
H49	6·35	—	—	36·7	5·8	—
I168	7·28	—	—	44·7	6·1	—
Q367	7·36	—	—	44·3	6·2	—
1514	8·11	0·402	0·049	39·3	4·9	98
G816	8·43	—	—	45·7	5·4	—
L242	8·64	—	—	52·5	6·1	—
		Mean	0·051		6·1	

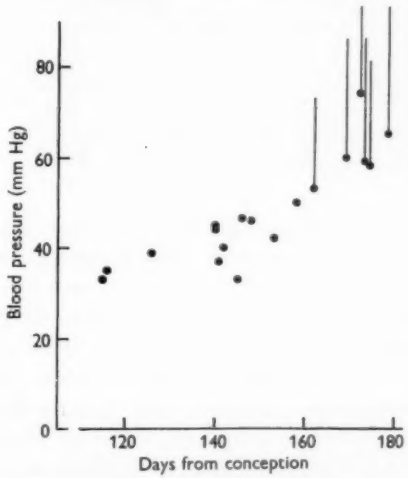


Fig. 10. The mean systemic arterial blood pressure plotted against age from conception for twelve foetal monkeys and for six infant monkeys 8–12 days after natural delivery (●). Both carotid arteries were occluded in the infants and the vertical lines indicate the rises of blood pressures observed.

had the least conceptual age (162 days) and the greatest age from birth (12 days) had the lowest blood pressure of the six infants. Occlusion of either carotid artery caused a rise of blood pressure. Simultaneous occlusion of both carotids caused rises (shown as vertical lines in Fig. 10) which were always greater than the sum of the rises observed when either was occluded independently. The rises of pressure on occlusion of both carotids were 21–48% of the initial blood pressure. Bilateral section of the carotid sinus nerves in three monkeys caused a rise of blood pressure and abolished the rise of pressure on carotid occlusion. The experiment on the monkey (No. 101) with the least conceptual age has been used to illustrate this point (Fig. 11).

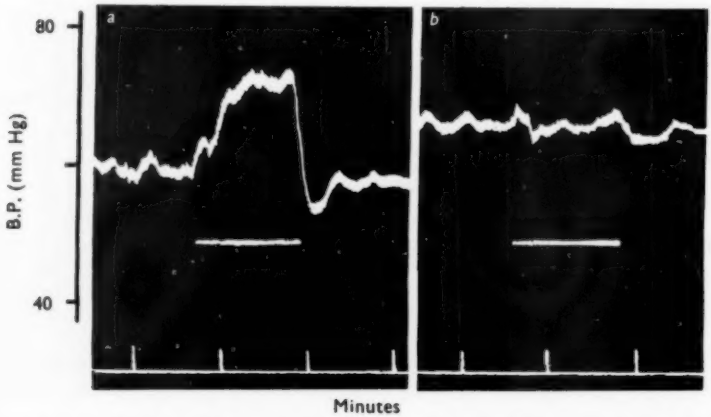


Fig. 11. Monkey No. 101, 395 g, born naturally at 150 days gestation age 12 days previously, pentobarbitone anaesthesia. Occlusion of both carotid arteries, indicated by the horizontal line, caused a rise of blood pressure (*a*). Denervation of both carotid bifurcations, between *a* and *b*, caused a rise of pressure, and occlusion of both carotids then had no effect on the blood pressure (*b*).

In two monkeys the Hering–Breuer respiratory reflexes were investigated. When the intratracheal pressure was abruptly increased (by attaching the tracheal cannula to a 500 ml. reservoir in which the pressure was above atmospheric) breathing slowed and became deeper, or ceased; the minimal effective pressure change was +5 to +7·5 cm water. When the intratracheal pressure was decreased, breathing became rapid and shallow; the minimal effective pressure change was −5 cm water. When both cervical vagi were cut the respiratory rate fell from about 90 breaths/min (which is normal for an infant monkey) to 30–40 breaths/min, and became much deeper; there was no change in blood pressure. Vagotomy abolished the response to raising the intratracheal pressure, and very greatly reduced the response to lowering the intratracheal pressure. Stimulation of the peri-

pheral end of the left vagus caused a profound fall of blood pressure and heart rate (Fig. 12).

The foramen ovale, ductus arteriosus and ductus venosus. In all six infant monkeys, 8–12 days old, the foramen ovale was anatomically patent at post-mortem examination. Comparison with specimens from foetal monkeys suggested that anatomical closure had not begun. Nevertheless, during life the arterial blood was well oxygenated, with a saturation of 93–94 %

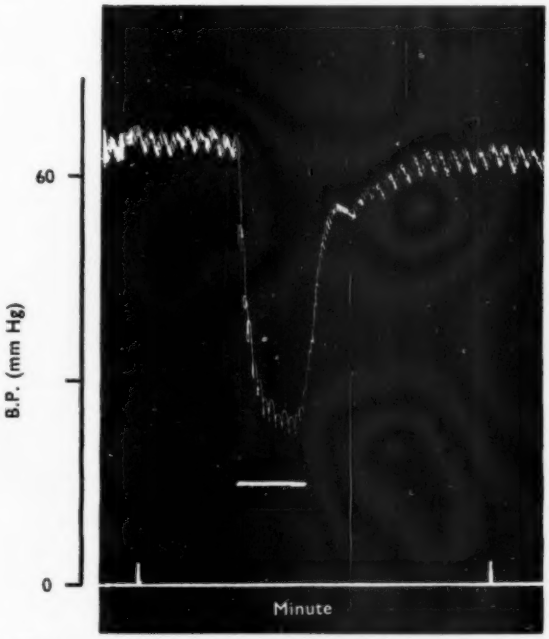


Fig. 12. Monkey No. 94, 517 g, born naturally at 168 days gestation age 10 days previously, pentobarbitone anaesthesia. Stimulation of the peripheral end of the left vagus nerve, indicated by the horizontal line, caused a profound fall of blood pressure and heart rate.

in the two monkeys in which measurements were made (Table 1), so that very little blood can have been passing through it. The ductus arteriosus was closed, and a fine probe could not be passed. Each monkey was examined during the first day of life with an ordinary stethoscope. In contrast to new-born lambs, calves and foals, no murmur was heard. However, in the monkeys the heart rate was high, 200–250 beats/min, and the chest was small. The ductus venosus was anatomically closed in all the infants; there was a small puckering in the portal sinus which marked the point of junction. The ductus venosus was patent in all the foetuses.

Acute asphyxia and anoxia

Figure 13 shows that the time between the onset of acute asphyxia or anoxia and the last gasp decreased from over 20 min at about 120 days' gestation age to less than 10 min at term. Foetal monkeys were asphyxiated by tying and cutting the umbilical cord on delivery; they were prevented from inhaling air by placing a rubber bag filled with saline over their heads. Figure 13 also includes a number of observations previously made by one of us (H.N.J.) in which the foetuses were asphyxiated by

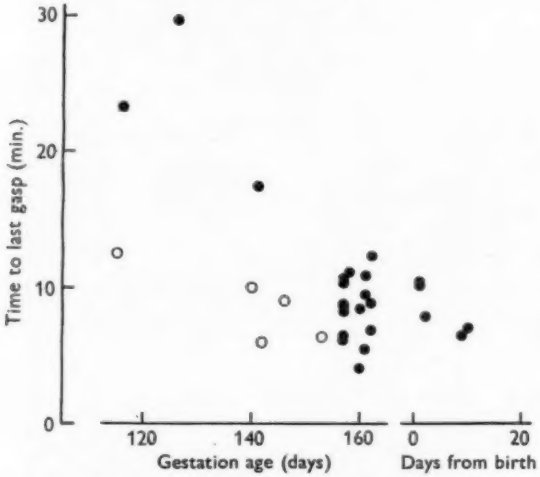


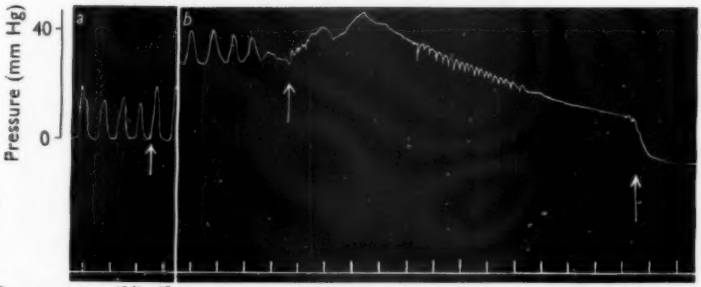
Fig. 13. Time interval between the onset of asphyxia or anoxia and the last gasp in foetal and new-born monkeys of different ages. Foetal monkeys were either delivered within the amniotic sac, or had a saline-filled bag placed over the head at delivery, and the cord was clamped and cut immediately (●). Infant monkeys were given nitrogen to breathe (●). Five foetal monkeys were also asphyxiated by rapidly opening the chest and heart on delivery (○).

delivery from the mother of the intact amniotic sac, in which the infants were kept until all respiratory efforts had ceased. New-born monkeys were made anoxic by exposure to 100% nitrogen.

In five unasphyxiated foetuses the chest was at once opened on delivery and a piece of cardiac ventricle was removed. The circulation was thus rapidly brought to a halt and the interval of time to the last gasp (Fig. 13, ○) was less than when anoxia was produced without arresting the circulation (Fig. 13, ●).

Figure 14 illustrates the course of events when the umbilical cord was tied in a foetus of 141 days' gestation age. The left-hand part of this figure (*a*) shows the pressure changes within the uterus recorded from a balloon.

The manometer was then transferred to a catheter in a femoral artery (*b*) and after four further uterine contractions had been observed the foetus was delivered, with a bag over his head, and the umbilical cord was tied at the second arrow. There was an abrupt rise of pressure of 5 mm Hg, followed by a more gradual further rise. The heart rate fell rapidly from 150 to 40–60 beats/min. Respiratory efforts began 25 sec after tying the cord and continued, becoming very rapid, for a little more than a minute. They ceased just over 2 min after tying the cord, and there was complete apnoea for the next 7½ min. A succession of slow deep gasps then began, each of which was associated with a small fall of blood pressure (Fig. 14).



Arterial O_2 saturation (%)	63	2
Blood glucose (mg/100 ml.)	27	151
Blood lactate (mg/100 ml.)	10	78

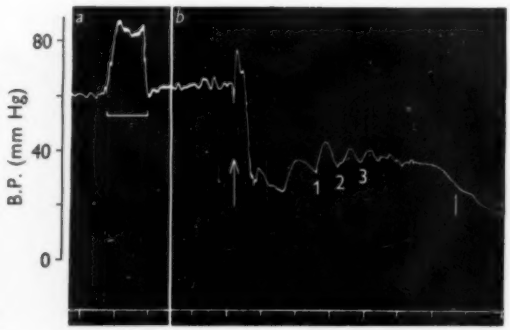
Fig. 14. Monkey No. 112, 360 g, 141 days gestation age, pentobarbitone anaesthesia. Intra-uterine pressure was recorded from a balloon in the amniotic sac (*a*), and a blood sample was taken from a catheter in a femoral artery at the first arrow. The manometer was then attached to the catheter to record foetal arterial pressure (*b*). The foetus was delivered, a saline-filled bag was placed over its head, and the cord was clamped and cut at the second arrow. Note in (*b*) the slow rise and gradual decline in blood pressure, with gasps which caused small brief falls in pressure. At the third arrow the chest was opened, a blood sample was removed from the heart and a piece of the ventricle was taken for analysis. Time marker, 2 min.

These finally ceased 17½ min after tying the cord, the blood pressure and heart rate gradually fell and at the third arrow the chest was opened and a sample of cardiac ventricle was removed. During the period of asphyxia the blood lactate rose from 10 to 78 mg/100 ml. and the blood glucose from 27 to 151 mg/100 ml.

The behaviour of foetus No. 110 of 145 days gestation age, which had been hypoxic for at least 1½ hr *in utero*, was somewhat different. When her cord was tied on delivery there was a small rise of blood pressure which lasted less than 1 min. She started gasping at about 4 min and finally ceased only 7½ min after the cord was tied. The heart was removed at 10¾ min and contained only 0·2 mg carbohydrate/g, the lowest recorded.

During the period of anoxia the blood glucose rose from 65 to 150 mg/100 ml. and the lactate from 121 to 155 mg/100 ml.

In contrast, Fig. 15 illustrates the effect of anoxia in a monkey 9 days after birth. In the left-hand part of this figure (*a*) both carotids were occluded and there was a large rise of blood pressure; the carotid sinus reflexes were therefore functional. At the arrow in (*b*) the tracheal cannula was attached to a long, wide tube through which nitrogen was slowly running to waste. There was a brief rise of blood pressure followed by a



Arterial O_2 saturation (%)	94	2
Blood glucose (mg/100 ml.)	48	14
Blood lactate (mg/100 ml.)	9	51

Fig. 15. Record of femoral arterial pressure in monkey No. 107, 561 g, born naturally at 165 days gestation 9 days previously, pentobarbitone anaesthesia. Occlusion of both carotid arteries, indicated by the horizontal line, caused a rise of blood pressure (*a*). An arterial blood sample was taken between *a* and *b*. At the arrow the tracheal cannula was attached to a long wide tube through which nitrogen was flowing. After a short while blood pressure fell precipitously and breathing ceased. After more than 2 min anoxia the monkey began to take a series of deep gasps (1, 2, 3, etc.) which gradually became feebler; the time of the last is indicated by the vertical line. A final blood sample was taken at the end of the record. Time marker 1 min.

profound fall, bradycardia and defaecation. Respiratory efforts increased in frequency for about 0·75 min and then temporarily ceased. A series of deep gasps (of which the first three are denoted by numerals in Fig. 15) began 2¼ min after the onset of anoxia, became more frequent and shallow and finally ceased at 6½ min (marked by the vertical line). Each of these gasps was followed by a rise of blood pressure. During the period of anoxia the blood lactate rose from 9 to 51 mg/100 ml., but in contrast to the foetus, the blood glucose fell from 48 to 14 mg/100 ml. The difference between the cardiovascular responses of the foetal and new-born monkeys to O_2 lack was particularly striking.

Observations were also made on the carbohydrate and lactate content of various tissues removed from monkeys after asphyxia, for comparison with those from unasphyxiated monkeys of similar ages. As Fig. 16 (○) shows, the carbohydrate content of the ventricles in unasphyxiated monkeys fell with increasing age, approximately to the same extent as did the survival time to the last gasp during asphyxia or anoxia (Fig. 13). The carbohydrate content of the ventricles fell to low values during O_2 lack (Fig. 16, ●). The lactate concentration in the blood and tissues (heart, lung, liver, and in the new-born, skeletal muscle) rose in anoxia, but the increase in cardiac lactate was always greater than that in the other tissues.

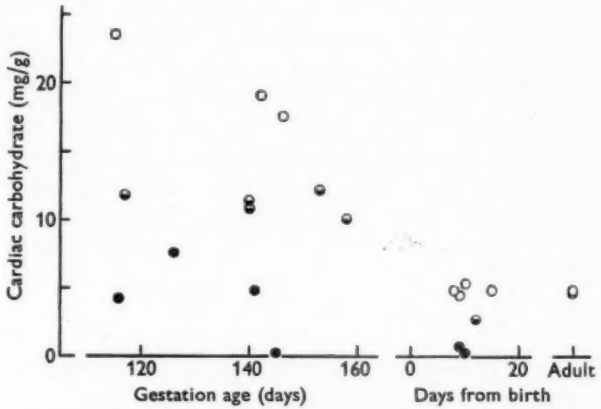


Fig. 16. The carbohydrate concentration in the cardiac ventricles plotted against age for monkeys which were not asphyxiated (○), and for others which were partially asphyxiated (◒) or severely asphyxiated (●).

Whereas there was always a large fall in cardiac carbohydrate during anoxia, the changes in carbohydrate concentration in lung and muscle were of doubtful significance. There was a large reserve of liver carbohydrate (37–73 mg/g) in both foetal and new-born monkeys, even at the end of asphyxia or anoxia. The hyperglycaemia observed in foetal monkeys suggests that part of this reserve was liberated during anoxia. However, the figures for the total liver carbohydrate in unasphyxiated foetal monkeys covered so large a range (60–104 mg/g) that it was not possible to demonstrate a significant fall in anoxia.

The changes in tissue carbohydrate with age will be described elsewhere, but it may be noted that there was a decrease in lung carbohydrate concentration between 115 and 140 days gestation from about 15 to 5 mg/g, and a further fall after birth. There were also considerable changes

in skeletal muscle carbohydrate; the level was high near term (29 mg/g) and fell to near adult values after birth (10 mg/g). There was little carbohydrate in the placenta from 140 days gestation onwards (< 3 mg/g). Histological examination of the tissues suggested that their glycogen content was proportional to their total carbohydrate content.

DISCUSSION

The foetal monkey

One of the problems in foetal physiology has been to determine and to provide some quantitative indication of normal physiological conditions, since hypoxia is so readily produced. In foetal lambs hypoxia caused a decrease in O_2 consumption, accumulation of blood lactate and a fall in blood pH when the femoral arterial O_2 saturation was reduced below 50% (Acheson, Dawes & Mott, 1957; Dawes & Mott, 1959*a*). The normal value in lambs near term under barbiturate anaesthesia was nearly 60%. This figure is much larger than that usually recorded by obstetricians from the human umbilical artery at delivery, which only rarely exceeds 40%. This discrepancy might have been due either to the experimental conditions or to species differences, for the uterus and placenta of the sheep are different from those of the primate. The fact that the femoral arterial O_2 saturation of the foetal rhesus monkey *in utero* was on the average 58%, with a blood lactate concentration as low as that in well oxygenated new-born monkeys, suggests that the observations on monkeys and lambs gave a truer picture of the normal intra-uterine environment than did those on the human foetus after delivery. The sheep uterus does not contract violently on delivery of the foetus by Caesarean section, nor is the placenta rapidly expelled, and it is therefore possible to make prolonged observations on the foetal lamb under good physiological conditions after delivery from the uterus. This is not possible in the monkey, in which the foetus rapidly becomes hypoxic on delivery. The observations of James, Weisbrot, Prince, Holaday & Apgar (1958) are consistent with the view that new-born human infants are almost always asphyxiated to some degree by the process of delivery, since the blood pH and buffer base rose progressively as they recovered during the first 24 hr after birth. Thus in primates it would seem that the only trustworthy evidence as to the normal intra-uterine environment is that obtained while the foetus is still *in utero*.

The changes in intra-uterine pressure during uterine contractions were within the limits of those observed by Ramsey, Corner, Long & Stran (1959) in rhesus monkeys at different conceptual ages, becoming greater after the 140th day. At or after the peak of a myometrial contraction (of

about 30 mm Hg) there was a small fall in foetal arterial O_2 saturation. An explanation for this may be that the uterine veins were compressed during a contraction, venous blood was dammed up in the placental pool, the arteriovenous pressure difference was temporarily decreased and uterine flow was reduced. These changes would have the effect of decreasing the mean O_2 content of blood in the intervillous spaces, and hence foetal O_2 saturation. There is evidence in other species that some such circulatory changes may occur in labour (Woodbury, Hamilton & Torpin, 1938; Woodbury, Hamilton, Abreu, Torpin & Fried, 1944; Caldeyro-Barcia, 1957; Hendricks, 1958; Assali, Dasgupta, Kolin & Holms, 1958).

Our knowledge of the course of the circulation in the foetus has relied very much upon experiments in artiodactyls. Huggett (1927) showed that the oxygen content of blood in the carotid artery in foetal goats exceeded that in the umbilical artery, indicating that mixture of the blood streams within the heart was incomplete. These findings were confirmed in sheep by Barcroft, Barron, Cowie & Forsham (1940). In seventeen mature lambs the carotid blood had an O_2 saturation which was on the average 6% (absolute) greater than that in the femoral artery, and this difference was independent of the level of the O_2 saturation between 30 and 80% (Dawes, Mott, Widdicombe & Wyatt, 1953; Dawes, Mott & Widdicombe, 1954). This is very similar to the observations in foetal monkeys, where the mean difference in O_2 saturation was 9%.

In the foetal monkeys arterial blood samples were obtained either from a femoral or brachial artery (with only the limb delivered from the uterus) or from a carotid artery dissected within the amniotic cavity. Reynolds, Paul & Huggett (1954) and Chinard, Danesino, Hartmann, Huggett, Paul & Reynolds (1956) used another method which at first sight has great advantages. According to them, 80% of rhesus monkeys have a double placenta. The vessels between the two placentas are outside the amniotic sac and can therefore be catheterized without loss of amniotic fluid or interference with the foetus. However, after our admittedly limited experience we would not recommend this method. In only seven out of the twelve foetuses was the placenta double, and in these the second placenta weighed 35–59% of the total. Ligature of an artery of a size sufficient to catheterize might therefore exclude a very considerable proportion of the placenta from the circulation; the exact proportion could only be determined at the end of the experiment.

In monkeys, as in sheep, goats, rabbits, guinea-pigs and rats (Barcroft, 1946), there was very little increase in placental weight during the last third of gestation, in contrast to the large increase in foetal weight (Fig. 3). The arterial O_2 saturation and the oxygen-carrying capacity of

the blood also appeared to have reached a steady level during this period, although more observations nearer term would be desirable. In these respects the monkey is similar to the lamb under barbiturate anaesthesia (Born, Dawes & Mott, 1955, 1956). In lambs there was a rapid fall in oxygen-carrying capacity after birth, from over 15 ml./100 ml. down to about 10 ml./100 ml. In the monkey the change was very much smaller (Fig. 2).

As Chinard *et al.* (1956) found, there were negligible quantities of fructose in the blood of foetal rhesus monkeys. The blood–glucose concentration was low, though over twice that in foetal lambs, and rose after birth, as in lambs (Shelley, 1960). Hypoxia or acute asphyxia caused a large increase in blood glucose in foetal monkeys. Similar observations have occasionally been made in foetal lambs, but the *immediate* effect of acute anoxia in the latter was to cause a fall in blood glucose (Dawes *et al.* 1959).

The new-born monkey

At birth the rhesus monkey is covered with a thin fur, it grasps its mother firmly and is warmed by contact with her. During the first day or two it crawls about its incubator. By the end of a week its movements are well co-ordinated and it can move its head fairly steadily. Rhesus monkeys normally show signs of hunger within 12–24 hr of birth and suck vigorously. In a cool environment they shiver, and can thus double their O_2 consumption, as do lambs. Human babies also can double their O_2 consumption under similar circumstances (Brück, Brück & Lemtis, 1958). However, new-born monkeys are small animals (only about 450 g at birth as compared with a 2·5 kg human baby or a 4 kg lamb) and so although at 22–24° C their peak O_2 consumption may be as high as 25 ml./kg.min within one day of birth, rectal temperature may yet fall considerably. For comparison, a new-born lamb will maintain its rectal temperature above 37° C in a similar environment with an O_2 consumption of only about 15 ml./kg.min. Calculation from the figures of Brück *et al.* shows that the peak O_2 consumption of mature new-born human infants at 23° C is less than 10 ml./kg.min; the rectal temperature of the very young babies fell below 35° C under these conditions.

In anaesthetized foetal sheep the rate of O_2 consumption per unit body weight was about the same as that of anaesthetized adult ewes (Dawes & Mott, 1959*a*). In the human infant after birth the *minimal* rate of O_2 consumption per unit body weight (i.e. at 32–35° C) was also about the same as that of an adult, judging from the observations of Brück *et al.* (1958). Unanaesthetized monkeys had a mean *minimal* O_2 consumption, measured at 35° C as soon after birth as possible, of 10·7 ml./kg.min. This is considerably greater than the figure of 6·1 ml./kg.min (Table 2)

for anaesthetized adult female monkeys. It remains to be seen whether this difference can be ascribed to anaesthesia. It is interesting to note that the neutral temperature of the new-born monkey, at which O_2 consumption is minimal, is less than that to which the foetus is exposed *in utero*.

In puppies the calculated metabolic rate at the neutral temperature (about 33° C) increased more than threefold over the first 3–4 days after birth (Gelineo, 1954). In lambs, the rate of O_2 consumption at the neutral temperature increased nearly threefold during the first day of life (Dawes & Mott, 1959*a*); in monkeys it increased nearly twofold over the first week and this was associated with a steady rise in rectal temperature over the first 3–4 days. The lambs were in a very cold environment, usually well below 20° C; the monkeys were kept in an incubator at 30° C. In both species the rate of O_2 consumption per unit surface area in a neutral environment increased to a figure which was almost identical with that of adult females. We have no quantitative information as to the thermal insulation of either infants or adults. Nevertheless, it is tempting to attribute the increase in O_2 consumption of the infants to the fact that after birth they were placed in an environmental temperature considerably below that to which they were exposed *in utero*. The observations of Brück *et al.* (1958) suggest that there was no increase in the metabolic rate of human infants at 32–35° C during the first week from birth, but their figures are few in number and more observations are now desirable in view of the animal data.

The effect of partial lack of O_2 upon O_2 consumption was similar to that described in new-born kittens and adult guinea-pigs (Hill, 1959), in young lambs (Cross, Dawes & Mott, 1959) and in rabbits (Adamsons, 1959). Thus in the neutral environment (at 35° C for infant monkeys) a reduction of O_2 content of the inspired air to as low as 10 % caused no change in O_2 consumption, whereas in a cold environment (in which O_2 consumption was increased) there was a fall. Cross, Tizard & Trythall (1958) observed a 17 % fall in the O_2 consumption of full-term human infants when they were given 15 % O_2 to breathe. It is uncertain whether these human infants were below their neutral environmental temperature. There is in any event a quantitative difference between new-born monkeys and human babies, because in the former the O_2 content of the inspired air had to be reduced below 13 % before there was a consistent fall in consumption.

The blood pressure rose with increasing gestation age in the monkey to reach a mean value of about 55 mm Hg at birth. In the adult monkey it is usually 100 mm Hg or more. The lamb has a rather higher pressure at birth, about 65 mm Hg (Dawes, 1956). As in lambs and in rabbits (Dawes & Mott, 1959*b*; Downing, 1960) a few of the principal cardiovascular

and respiratory reflexes were shown to be active after birth. The absolute rise of systemic pressure on occlusion of both carotid arteries in infant monkeys was, of course, much less than that seen in adult monkeys (Heymans, 1958: *Papio cinocephalus* and *Cercocebus albigenae*), but relative to the initial pressure it appears very similar (as judged by Heymans's illustrations).

Acute asphyxia and anoxia. The effect of acute anoxia upon infant monkeys was surprising. Whereas in the foetal monkeys asphyxia had produced a slow, well maintained rise in blood pressure accompanied by an increase in blood glucose, in the infant monkeys there was only a transient rise in blood pressure which was followed within less than 1 min by an abrupt fall, and the blood glucose also fell. There were differences between the experiments which require consideration. The foetuses were asphyxiated by tying the umbilical cord, and one would therefore suppose that the CO_2 content of the blood and tissues would increase somewhat before the available oxygen was exhausted. The infant monkeys were given nitrogen to breathe, and were thus able to excrete CO_2 so long as breathing continued. The period of observation, before the blood pressure fell to a very low value, was also greater in the foetus than after birth. Yet none of these considerations suggest a wholly satisfactory explanation for the difference between the behaviour of foetal and new-born monkeys. Swann, Christian & Hamilton (1954) found that, as in the infant monkeys, there was an immediate rapid fall of blood pressure in 3–4-day-old puppies given nitrogen to breathe; the blood pressure only partly recovered about 3 min later, the blood pH fell below 7·0 and the calculated pCO_2 rose to a very high value. In foetal lambs acute asphyxia caused a preliminary rise of blood pressure, as in the foetal monkeys (Dawes *et al.* 1959). In adult sheep and dogs acute anoxia also causes a very large rise of blood pressure, accompanied by a large increase in heart rate which was never seen in the foetal or new-born animals. The immediate fall of blood pressure on exposure to nitrogen therefore appears to be a characteristic of the new-born animal, according to the limited evidence yet available. In the monkey this cannot be ascribed to the absence of reflex control of the cardiovascular system.

The observations on the changes in blood and tissue carbohydrate and lactate concentrations during anoxia emphasize the importance of the initial cardiac glycogen reserves. The concentration of lactate in the heart after a period of anoxia was always greater than that in the blood or other tissues. Yet the increase in cardiac lactate represented only about one tenth of the carbohydrate lost from the heart (taking the mean cardiac carbohydrate and lactate in unasphyxiated monkeys of each age as the initial level). The calculated fall in cardiac carbohydrate was more than

sufficient to account for the total rise in cardiac and blood lactate (assuming that the blood volume was 6% of body weight; Gregersen, Sear, Rawson, Shu Chien & Saiger, 1959) if all the carbohydrate had been converted to lactic acid. In foetal lambs similar calculations also led to the conclusion that the loss of carbohydrate from the heart could account for a very high proportion of the rise in lactate in the blood and tissues (Dawes *et al.* 1959). As in lambs and other animals, the survival time of monkeys during asphyxia or anoxia fell with increasing age by approximately the same proportion as did the initial glycogen reserves of the heart.

The observations on foetus No. 110, which had been hypoxic for a long while *in utero*, were interesting for two reasons. First the blood lactate had risen gradually to 121 mg/100 ml.; when the cord was tied on delivery this foetus did not take its last gasp for a further 7½ min and the terminal blood and cardiac lactate levels were 155 mg/100 ml. and 5·2 mg/g, respectively. In the other foetal and new-born monkeys the blood lactate rose to only 51–91 mg/100 ml. and the terminal cardiac lactate was only 2·0–2·7 mg/g after a period of anoxia which was continued to beyond the last gasp; it is therefore unlikely that in them the lactate level was a limiting factor determining survival, though it is probable that changes in tissue pH, about which we have no information, are of importance. Secondly, in foetus No. 110 the interval of time between tying the cord and the last gasp was much reduced as compared with monkeys of the same gestational age which were not previously hypoxic (Figs. 13 and 14). The insidious nature of the effect of prolonged hypoxia *in utero* is only too apparent, for while the danger was obvious from the measurements of foetal blood pressure, arterial O_2 saturation, blood lactate and even blood sugar, none of these would normally be available to the obstetrician. The fall in heart rate was comparatively small until delivery. The experience with this unusual foetus provides a warning. Experiments on acute asphyxia represent only one of the hazards which may be encountered at birth. It is possible that many foetuses are subjected to considerable periods of hypoxia before final delivery, and their capacity to survive may thereby be dissipated.

SUMMARY

1. In foetal rhesus monkeys *in utero* under pentobarbitone anaesthesia the mean O_2 saturation of femoral arterial blood was 58%. Brachial arterial blood was on the average 9% higher. On delivery the uterus contracted strongly and the arterial O_2 saturation fell considerably, even though the umbilical cord was intact.

2. The foetal blood lactate concentration was similar to that in new-born monkeys, the blood glucose concentration rose after birth. The

oxygen-carrying capacity of the blood showed little change during the last third of gestation or after birth.

3. The weight of foetal monkeys doubled during the last third of gestation, while that of the placenta and membranes showed only a trivial increase.

4. New-born monkeys shivered and their rate of O_2 consumption was doubled when the environmental temperature was reduced from 35 to 24° C. The monkeys were kept in an incubator at 30° C and their rate of O_2 consumption *in the neutral environment* (at 35° C) almost doubled during the first 6–10 days after birth.

5. In infant monkeys 8–12 days old the rate of O_2 consumption under anaesthesia was more than twice that of adult females when expressed per unit of body weight, but was about the same when expressed per unit of surface area.

6. In infant monkeys a reduction in the O_2 content of the inspired air to 10–14% caused no change in O_2 consumption at an environmental temperature of 35° C, but at 22–26° C there was a fall.

7. In infant monkeys the Hering–Breuer inflation and deflation respiratory reflexes were present. The arterial blood pressure rose during gestation to reach about 55 mm Hg at birth. Occlusion of the carotid arteries caused a rise of pressure which was abolished by denervation of the carotid bifurcations. Stimulation of the peripheral end of the vagus caused a large fall of blood pressure and heart rate.

8. In monkeys 8–12 days old the foramen ovale was still (anatomically) open, though probably functionally closed *in vivo* as judged by the high arterial O_2 saturation. The ductus arteriosus and ductus venosus were both closed.

9. The effects of acute asphyxia and anoxia were compared in foetal and new-born monkeys. In young foetuses gasping continued for 20 min. or more and there was an initial gradual rise of blood pressure. Infant monkeys ceased gasping in 7–8 min and there was only a brief rise of blood pressure followed by a profound fall. The blood and tissue carbohydrate and lactate concentrations were measured, and were examined in relation to the decreased ability to withstand anoxia which was observed with increasing age.

10. The observations are discussed in relation to what is known of the behaviour of foetuses and new-born infants of other species, particularly in sheep and man.

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FORMATION AND CATABOLISM OF HISTAMINE IN CAT BRAIN *IN VIVO*

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Experiments *in vitro* have shown that brain tissue possesses mechanisms both for the formation and for the catabolism of histamine (White, 1959). Formation of ^{14}C-histamine from ^{14}C-histidine was demonstrated in brain tissue from cat, pig and dog and was highest in hypothalamus. Feline brain tissue also catalysed the catabolism of histamine, the major pathway being ring-N methylation of histamine. The present investigation was undertaken to find out whether these reactions, which are schematized in Fig. 1, also occur in the cat brain *in vivo*. The same isotope dilution technique was used as in the earlier *in vitro* experiments. This technique permits the use of low, probably pharmacologically inactive, concentrations of the radioactive substances, and provides information as to the relative importance of the reactions by which histamine is metabolized. The method does not give absolute values for the tissue content of histamine nor of its metabolites.

METHODS

Cats of either sex weighing 2·5–4·5 kg were anaesthetized with Na pentobarbitone (45 mg/kg I.P.) The trachea was cannulated, and a polyethylene tube was introduced into a femoral artery for the collection of blood samples. The cerebral ventricles were perfused from the lateral ventricle to the cisterna magna, as described by Feldberg & Sherwood (1953) and by Bhattacharya & Feldberg (1957, 1958*a*), with some modifications in the cannulation of the lateral ventricle. Through a skin incision part of the temporal muscle on one side was removed to free the parietal bone for trephining. The dura was exposed and punctured with a sharp needle at a point about 1 cm lateral to the mid line. The intraventricular cannula was introduced into the brain with some inclination towards the mid line. The cannula was prepared from a 'Record' type hypodermic needle (outer diameter 0·7 mm) with approximately 5 mm of nylon or polyethylene tubing (outer diameter approximately 1 mm) fitted to the tip; the tubing was provided with side holes and the lumen was obliterated at the tip. The animal's head was kept elevated by a holder. Communication between the cannula and the lateral ventricle was indicated by inflow of fluid varying synchronously with respiration and by an instantaneous acceleration of flow on entering the cisterna magna. The position of the cannula was confirmed by dissection of the brain after the experiment. The cannula was either left in place during the dissection, or its track could be clearly seen on the cut surface as a line of minute blood points in the white matter. In some of the experiments the tip of the cannula had penetrated 1 mm or less into the floor of the lateral ventricle. In a few preliminary preparations, not used for experiments with radioactive

substances, a dye was injected through the cannula in position. The entire lining of the cerebral ventricles was stained, including the opposite lateral ventricle, but no staining of the brain substance was observed along the track of the cannula. In no instance did bleeding occur in the cannulated ventricle. In two experiments a faint tinge of blood appeared in the initial portion of the effluent as a result of damage to the dorsal surface of the medulla oblongata on puncturing the atlanto-occipital membrane.

The cerebral ventricles were perfused with radioactive substances dissolved in Tyrode solution of the following composition (g/l.): NaCl 8·00, KCl 0·20, $CaCl_2 6H_2O$ 0·40, $MgCl_2 6H_2O$ 0·20, $NaHCO_3$ 1·00, and NaH_2PO_4 0·06. The infusions were made with a motor-driven syringe at a constant rate. The infusion rate was 0·11 ml./min with the exception of experiments No. 4 and 5 in Table 1 (0·08 and 0·23 ml./min, respectively), and the total volume infused

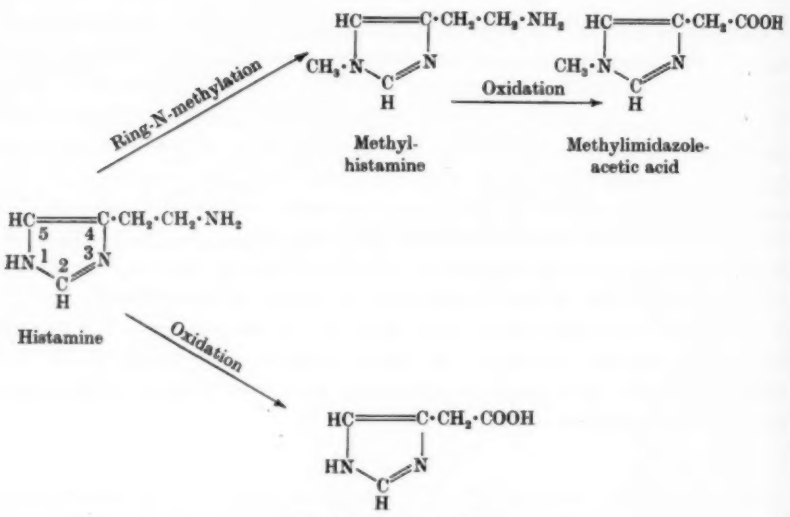

Fig. 1. Diagram to show the chemical inter-relationships between the radioactive substances determined. The substances were labelled with a ^{14}C-atom in the 2-position of the imidazole ring. For a full account of the various biological pathways of histamine catabolism see Schayer (1959).

was 4·5 ml. The effluent collected during the infusion exceeded this volume by some 10%. At the end of the perfusion with the radioactive substances the ventricles were perfused with 5 ml. of Tyrode solution at a rate of approximately 0·4 ml./min to wash out radioactive substances. For analysis the combined effluents were used. The whole brain including the cerebellum and the medulla oblongata down to the foramen magnum was excised (White, 1959).

The methods for measuring ^{14}C-histamine and its metabolites by isotope dilution technique have been described by Schayer and others and were similar to those used for the study on ^{14}C-histamine metabolism in brain *in vitro* (White, 1959). The brain tissue was minced, samples were taken for analysis, the respective carrier substances were added and the protein was precipitated with trichloroacetic acid. The brain samples were centrifuged three times and the combined supernatant fractions were collected, discarding the sediments.

Centrifugation proved more convenient than the filtration used earlier. The effluent was not treated with trichloroacetic acid and was not centrifuged; otherwise, it was prepared in the same way as the brain tissue.

Histamine was extracted into *n*-butanol (Kahlson, Rosengren, Westling & White, 1958) from an alkaline aqueous solution saturated with Na_2SO_4 and the butanol fraction was extracted with HCl. In the experiments where ^{14}C-histidine had been infused the butanol fraction was shaken with a solution of non-radioactive histidine before the extraction with HCl. The acid phase was evaporated to dryness; this residue consisted mainly of histamine dihydrochloride from which histamine dipicrate was prepared. For further purification pipsylhistamine was prepared and counted.

Methylhistamine was extracted into chloroform from an alkaline aqueous solution saturated with Na_2SO_4 (Rothschild & Schayer, 1958). After evaporation of the chloroform to dryness, the residue consisted mainly of methylhistamine dihydrochloride; methylhistamine dipicrate was prepared from it and counted.

Imidazoleacetic and methylimidazoleacetic acids were separated from histamine and methylhistamine on a cation exchange column (Dowex–50) (Lindell & Schayer, 1958*a*). Pipsylimidazoleacetic acid and the picrate of methylimidazoleacetic acid were prepared and counted. All determinations of imidazoleacetic acid were preceded by acid hydrolysis of the samples, and the figures for imidazoleacetic acid therefore denote the total amount, free and conjugated.

The total radioactivity in the blood was assayed in a manner similar to that used by Schayer & Cooper (1956) for urine. The samples were precipitated with trichloroacetic acid and filtered, and the trichloroacetic acid removed by ether. A suitable portion of the filtrate was transferred to a small plate, dried and then counted in the flow-counter. To compensate for self-absorption of radiation the following procedures were employed: (*a*) a plate was prepared with an 'infinitely thin' layer of ^{14}C-histamine and (*b*) the same amount of this ^{14}C-histamine was mixed with the sample and the mixture plated. The counts so obtained, together with the counts for the sample alone, were used to calculate the self-absorption, and the values for total radioactivity of ^{14}C-compounds in the blood were accordingly corrected. These figures for total radioactivity should be regarded as estimates rather than exact values, because the extraction of the blood may have been incomplete.

The measurements of the radioactivity of ^{14}C-histamine and its metabolites were made under conditions of infinite thickness in a gas-flow counter with a background activity of 23–25 counts/min. The criterion of purity was constant radioactivity after repeated recrystallizations. At least 900 counts were taken of each sample after each recrystallization, and 3–6 recrystallizations were needed to obtain constant radioactivity. Differences in counting rates smaller than those corresponding to 0·2–0·3% in Tables 1 and 2 are not significant.

For the incubations of cerebrospinal fluid with ^{14}C-histamine and ^{14}C-histidine see White (1959).

^{14}C-histamine dihydrochloride and ^{14}C-L-histidine, both labelled in the 2-position of the imidazole ring, were purchased from the Radiochemical Centre, Amersham, Bucks. All weights of the radioactive substances in the tables refer to the bases. For details of the ^{14}C-histamine see White (1959). The ^{14}C-L-histidine gave $9{\cdot}7 \times 10^7$ counts/min/mg base at zero thickness measured in the flow-counter, and 1 μg of ^{14}C-histamine derived from this ^{14}C-histidine gave, after addition of carrier histamine and precipitation in the usual manner, 6500 counts/min/μg base, measured at infinite thickness in the flow-counter. When subjecting the ^{14}C-histidine to the usual procedures for measurement of ^{14}C-histamine, small quantities of ^{14}C-histamine were invariably found. The amount of ^{14}C-histamine was proportional to the amount of ^{14}C-histidine extracted. It was found that 1·24 mg ^{14}C-histidine (i.e. one container as delivered by the manufacturer) contained 0·3 μg ^{14}C-histamine and that the solution of ^{14}C-histidine used for the perfusion experiments contained ^{14}C-histamine

in a concentration of 0·06 μg/ml. In each experiment with ^{14}C-histidine a sample of the infused solution was therefore analysed for ^{14}C-histamine.

Iproniazid was used in the form of 'Marsilid' base (Hoffmann-La Roche and Co. Ltd., Basle).

RESULTS

Histamine catabolism

It is known from experiments on conscious cats that large doses of histamine injected into the cerebral ventricles produce a variety of reactions (Feldberg & Sherwood, 1954), whereas smaller doses (15 μg) do not, apart from some licking movements. In the present investigation it was possible to use doses of radioactive histamine which were 'sub-threshold' in this respect and still large enough to contain radioactivity in amounts that could be conveniently assayed. It was found that 9 μg of ^{14}C-histamine in a concentration of 2 μg/ml. was suitable, and this was used in the majority of experiments.

TABLE 1. Catabolism of ^{14}C-histamine in cat brain during ventricular perfusions. Percentage of infused ^{14}C-histamine recovered as histamine and metabolites

Expt. no.	Infusion time (min)	^{14}C-histamine infused (μg)		Percentage recovered as: Histamine	Methylhistamine	Methylimidazoleacetic acid	Imidazoleacetic acid	Total recovery
1	40	9	Effluent	16	5·6	0·7	0·0	55
			Total brain	0·5	21	11	0·3	
2	40	9	Effluent	82	3·6	0·3	0·3	97
			Total brain	0·5	6·3	3·7	0·3	
3	40	9	Effluent	55	3·3	0·3	0·1	69
			Total brain	0·4	5·1	4·0	0·2	
4	60	9	Effluent	69	5·0	1·0	1·1	101
			Total brain	0·8	11	12	1·0	
5	20	7	Effluent	70	2·0	0·3	0·4	84
			Total brain	0·3	5·5	5·4	0·3	

As can be seen in Table 1, in most experiments the greater part of the infused histamine appeared unchanged in the effluent. Experiment 1 is exceptional in that little histamine was present in the effluent. On the whole rather large variations were observed in the histamine content of the effluents, and thus in the total recoveries. The reason for this is unknown; a possible explanation could be that the infused fluid escaped in varying amounts into some ventricular or subarachnoid space not thoroughly drained by the cannula in the neck (Bhawe, 1958). In similar experiments on histamine Bhattacharya & Feldberg (1958*b*) using a slightly larger concentration obtained recoveries during the first 40 min of the same order of magnitude as those encountered in the present study.

It is evident from Table 1 that under the conditions of these experiments methylation is the principal pathway for histamine catabolism in the living cat's brain, where only methylated ^{14}C-histamine derivatives are found. Little or no imidazoleacetic acid was formed.

In two experiments cerebrospinal fluid was collected from two normal animals under anaesthesia. The fluid (2 ml.) was incubated with ^{14}C-histamine (0·5 μg/ml.) in phosphate buffer solution at pH 7·4 for 3 hr in air at 37° C. After the incubation all the added ^{14}C-histamine was recovered. No methylhistamine, methylimidazoleacetic acid or imidazoleacetic acid could be demonstrated. It is therefore unlikely that the cerebrospinal fluid contributed to the catabolism of the infused ^{14}C-histamine.

It is unlikely that the radioactive substances introduced into the brain are absorbed by the blood, carried to other parts of the body and then returned to the brain in an altered chemical form as the systemic blood during and just before the termination of the perfusion contained little or no radioactivity. In none of the experiments could radioactive histamine, methylhistamine or methylimidazoleacetic acid be demonstrated in the blood. In some of the experiments a tendency was observed towards an increase in the radioactivity of the blood during the course of the experiment. It was therefore desirable to keep the duration of the experiments short. The highest value observed for the total radioactivity (assayed from 5 ml. portions) in the present series of experiments corresponded to about 0·01 % of the ^{14}C-histamine administered/ml. blood. ^{14}C-histamine has been shown to penetrate slowly or not at all from the blood into the brain in the rat (Halpern, Neveu & Wilson, 1959).

Influence of iproniazid on histamine catabolism

Iproniazid is an inhibitor of monoamine oxidase and is known to increase the content of some biologically active amines in the brain, such as 5-hydroxytryptamine and noradrenaline (for references see Brodie, Spector & Shore, 1959). It has been shown that iproniazid in intact cats changes the catabolism of injected ^{14}C-histamine, as judged by the urinary excretion of ^{14}C-compounds, so that the formation of a radioactive histamine metabolite, later identified as methylimidazoleacetic acid, is suppressed (Schayer, Kennedy & Smiley, 1953; Schayer, 1956). Iproniazid also inhibited the formation of ^{14}C-methylimidazoleacetic acid in the cat brain after the administration of ^{14}C-histamine.

In the first two experiments (Table 2) iproniazid was dissolved in the ventricular perfusion fluid together with the ^{14}C-histamine but did not cause significant changes in the pattern of histamine catabolism. In the third experiment a cat was injected on three successive days with iproniazid 100 mg/kg subcutaneously; this dose is similar to that used by Schayer *et al.* (1953). The ventricular perfusion started about 2 hr after the last injection. After this treatment the formation of methylimidazoleacetic acid in the brain was completely inhibited. This rather large dose of iproniazid seemed to be somewhat toxic to the animal. The cat lost in weight and appetite, and on operation the tissues appeared dehydrated and the blood showed brownish discoloration, similar to that given by methaemoglobin. It was therefore decided to perform a fourth experiment

similar to the third, but reducing the dose of ipronizaid to 25 mg/kg. In this cat no toxic symptoms were noticed, but the effect on the catabolism of the infused histamine was the same, i.e. no methylimidazoleacetic acid was formed in the brain.

Histamine formation

Histamine formation in the brain was demonstrated by perfusing the ventricles with ^{14}C-histidine and measuring the amount of ^{14}C-histamine in the brain and in the effluent after the perfusion. Parallel determinations

TABLE 2. Effect of iproniazid on histamine catabolism in cat brain during ventricular perfusions with 9 μg ^{14}C-histamine for 40 min. Percentage of infused ^{14}C-histamine recovered as histamine and metabolites

Expt. no.			Percentage recovered as: Histamine	Methylhistamine	Methylimidazoleacetic acid	Imidazoleacetic acid	Total recovered
1	Perfusion fluid contained iproniazid 0·2 mg/ml.	Effluent	73	4·3	0·2	0·2	89
		Total brain	0·4	6·9	4·0	0·1	
2	Perfusion fluid contained iproniazid 2 mg/ml.	Effluent	70	5·0	0·2	0·3	93
		Total brain	0·8	12	4·6	0·4	
3	100 mg iproniazid/kg subcutaneously daily for 3 days	Effluent	60	3·1	0·0	0·0	77
		Total brain	0·5	13	0·0	0·0	
4	25 mg iproniazid/kg subcutaneously daily for 3 days	Effluent	21	4·3	0·0	0·0	37
		Total brain	0·4	11	0·0	0·0	

of ^{14}C-methylhistamine were made. The results are shown in Table 3 (Expts. 1–3). In these experiments the hypothalamus was analysed separately because this part of the brain had shown a greater histamine-forming capacity *in vitro* than other parts (White, 1959). It is again evident from Table 3 that the hypothalamus contained higher concentrations of ^{14}C-histamine than the rest of the brain.

As mentioned in Methods the preparation of ^{14}C-histidine used in these experiments contained an admixture of ^{14}C-histamine. Although the content of ^{14}C-histamine was relatively small it was not necessarily unimportant because the percentage conversion to ^{14}C-histamine of the rather large quantities of ^{14}C-histidine used was small. To evaluate the significance of the ^{14}C-histamine found in the brain, experiments were designed to establish whether the ^{14}C-histamine infused in these experiments could account for the ^{14}C-histamine found in the brain. In Expts. 4 and 5 of Table 3 a solution was infused that contained the same amount of

^{14}C-histamine (0·06 μg/ml.) as that present in the ^{14}C-histidine solutions of Expts. 1–3 of Table 3. In Expt. 4 the perfusion fluid contained, in addition, non-radioactive L-histidine in the same concentration as that of the radioactive histidine in Expts. 1–3. It was found that this low concentration of ^{14}C-histamine was not sufficient to lead to the appearance of ^{14}C-histamine or of ^{14}C-methylhistamine in the brain in significant quantities. It was concluded that the ^{14}C-histamine found in the brain after perfusion with ^{14}C-histidine represented a true formation of histamine in the brain during the experiment.

TABLE 3. Histamine formation in cat brain during ventricular perfusions with ^{14}C-histidine for 40 min. The control Expts. 4 and 5 show the effect of a small amount of added ^{14}C-histamine (see text). The figures for ^{14}C-histamine and ^{14}C-methylhistamine denote ng/g tissue in the hypothalamus and in the rest of the brain, and ng in the total volume of effluent

Expt. no.	Solution infused		^{14}C-histamine (ng/g)	^{14}C-histamine (ng)	^{14}C-methylhistamine (ng/g)	^{14}C-methylhistamine (ng)
1	^{14}C-histamine 0·25 mg/ml.	Hypothalamus	20	—	—*	—
		Rest of brain	2	—	1	—
		Effluent	—	270	—	27
2	^{14}C-histidine 0·25 mg/ml.	Hypothalamus	12	—	4	—
		Rest of brain	<1	—	<1	—
		Effluent	—	240	—	15
3	^{14}C-histidine 0·25 mg/ml.	Hypothalamus	29	—	12	—
		Rest of brain	1	—	1	—
		Effluent	—	240	—	19
4	^{14}C-histamine 0·06 μg/ml. + non-radioactive histidine 0·25 mg/ml.	Hypothalamus	0	—	0	—
		Rest of brain	0	—	0	—
		Effluent	—	160	—	15
5	^{14}C-histamine 0·06 μg./ml.	Hypothalamus	0	—	0	—
		Rest of brain	0	—	0	—
		Effluent	—	150	—	11

* Sample lost.

Blood samples collected in the experiments in which ^{14}C-histidine was infused were more radioactive than those collected in the preceding section when ^{14}C-histamine was infused. This is not surprising, as the amount of radioactivity infused in the form of ^{14}C-histidine was about 250 times greater. The radioactivity found in the different experiments corresponded to approximately 0·005–0·01 % of the infused ^{14}C-histidine per ml. blood, assayed from 5 ml. portions. In none of the blood samples could ^{14}C-histamine or ^{14}C-methylhistamine be detected.

In one experiment cerebrospinal fluid was collected from a normal animal under anaesthesia. The fluid (2 ml.) was incubated with buffer solution and ^{14}C-histidine (10 μg/ml.) for 3 hr in air at 37° C. The content of ^{14}C-histamine in the sample after the incubation was the same as that of a blank incubated simultaneously. It was concluded that the cerebrospinal fluid is unable to form histamine.

DISCUSSION

The present experiments *in vivo* confirm the results obtained *in vitro* (White, 1959) and show that ring-N methylation is the major pathway for histamine catabolism in the cat's brain. On the other hand, the present experiments show a proportionally greater oxidation of methylhistamine, i.e. formation of methylimidazoleacetic acid. A purified enzyme catalysing ring-N methylation of histamine has been prepared from mouse liver by Lindahl (1958) and from several mammalian tissues, including cat brain, by Brown, Tomchick & Axelrod (1959), who named this enzyme imidazole-N-methyl transferase. The methylation of histamine observed in the present experiments might conceivably be due to the action of such an enzyme which appeared to methylate practically all radioactive histamine in the brain tissue. The question arises whether this methylation converts this biologically active substance into another also active, or whether it merely reduces the amount of active material by converting it into an inert substance. This question cannot be settled until more is known about the effect of methylhistamine on brain function. No major difference in methylating capacity has been observed *in vitro* between different parts of the cat brain (Brown *et al.* 1959; White, 1959).

Several studies indicate that the oxidation of methylhistamine to form methylimidazoleacetic acid is effected by an enzyme resembling monoamine oxidase (Davison, 1958; Schayer, 1959), and it seems reasonable to assume that this is also true in the present experiments. The effect of iproniazid provides supporting evidence. Iproniazid injected subcutaneously abolishes the formation of methylimidazoleacetic acid from infused radioactive histamine, but it does not produce a pronounced effect on the amount of radioactive histamine found in the brain, because the histamine is, in fact, still almost completely methylated. On the other hand, it seems that endogenous brain histamine has a fate different from histamine introduced into the brain by ventricular perfusion. In the experiments on histamine formation from radioactive histidine, less radioactive methylhistamine was found in proportion to radioactive histamine than in the experiments in which radioactive histamine was infused. The newly formed histamine in the living brain may be less readily attacked by the methylating enzyme than is administered histamine. Another, less likely, explanation for the small amount of methylhistamine could be that the methylhistamine is very rapidly converted to methylimidazoleacetic acid; this substance could not be determined in the experiments with radioactive histidine because of the limitations of the method, as discussed by Lindell & Schayer (1958*b*).

The catabolism of radioactive histamine in the brain was not greatly

affected by iproniazid infused into the ventricles, whereas a complete inhibition of formation of methylimidazoleacetic acid resulted from subcutaneous injections of iproniazid. One reason for this difference may be that the doses of iproniazid were too low; on the other hand, the highest concentration used was about 100 times greater than that known to inhibit monoamine oxidase *in vitro* (Davison, 1958). Possibly iproniazid does not penetrate into the brain from the ventricular fluid rapidly enough to become effective in these experiments of rather short duration, or it may require a longer contact with the enzyme to produce inhibition.

The present results provide little information on the location within the brain of the substances and reactions studied. It is probable that the structures near the ventricles come into closer contact with the perfusion fluid and therefore contain more of the radioactive substances. It does not therefore seem justified to conclude from the present experiments that the hypothalamus is more active in forming histamine than other parts of the brain, but only that the present findings are consistent with a high histamine-forming capacity there, as already found *in vitro* (White, 1959).

High concentrations of monoamine oxidase are present in some regions of the mammalian brain, including the ependyma (Arioka & Tanimukai, 1957). However, it is unlikely, that the formation of methylimidazoleacetic acid in the present experiments took place in the very walls of the cerebral ventricles, because the formation of methylimidazoleacetic acid was unaffected when iproniazid was given intraventricularly and because no methylimidazoleacetic acid escaped into the perfusion fluid.

SUMMARY

1. In anaesthetized cats the cerebral ventricles were perfused with radioactive histamine. Most of the infused histamine appeared in the effluent, but part of it entered the brain substance.

2. It was found that in the brain histamine was converted to methylhistamine (1-methyl-4(β-aminoethyl)-imidazole) and methylimidazoleacetic acid (1-methyl-imidazole-4-acetic acid), showing that ring-N methylation is the principal pathway for histamine catabolism in the cat's brain.

3. The formation of methylimidazoleacetic acid was inhibited by iproniazid injected subcutaneously. No inhibition occurred when iproniazid was infused into the cerebral ventricles together with the histamine.

4. Histamine formation in the brain was demonstrated by the appearance of radioactive histamine in the brain after ventricular perfusion with radioactive histidine.

5. The possibility is considered that in the brain the catabolism of

newly formed histamine is different from that of histamine administered to the brain.

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THE ANTAGONISM BETWEEN TUBOCURARINE AND SUBSTANCES WHICH DEPOLARIZE THE MOTOR END-PLATE

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It is well known that acetylcholine (ACh) depolarizes the end-plate region of skeletal muscle, and that this action can be blocked by tubocurarine (Cowan, 1936; Kuffler, 1943). By using the contracture response of the frog's rectus as a measure of drug action, several workers (van Maanen, 1950; Kirschner & Stone, 1951; Ariëns, van Rossum & Simonis, 1956) have studied this antagonism quantitatively, concluding that these and several related substances compete for receptors at the end-plate, as originally proposed by Langley (1905). In the present work the action of tubocurarine has been examined by the more direct method of determining the effect of known concentrations on the end-plate depolarizations evoked by bath-applied ACh or carbachol. Extracellular recording was used for the most part, but in some experiments micro-electrodes were employed to measure the responses of single fibres. In this way it was found that individual end-plates have similar affinities for tubocurarine.

METHODS

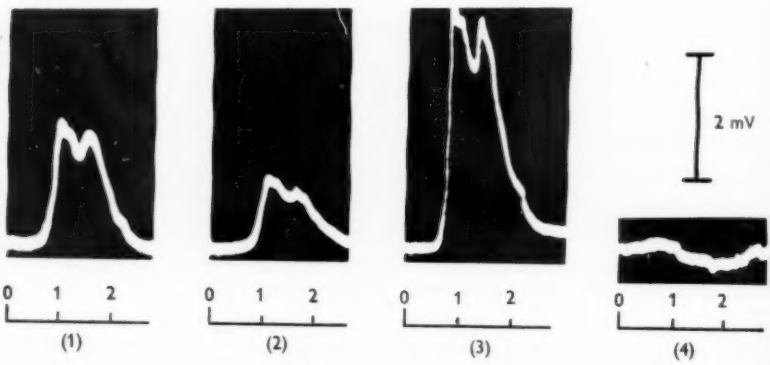
The sartorius, ext. long. dig. IV and rectus abdominus muscles of *Rana temporaria* were used throughout the year, at room temperature (19–23° C) unless stated to the contrary. Some experiments were made on nerve–sartorius preparations and others on chronically denervated muscle.

Procedure with external electrodes

In much of the present work a liquid–air interface was used as an extracellular electrode with which to measure the response of the end-plate region of the m. ext. long. dig. IV to depolarizing agents (Fatt, 1950). Typical records obtained in this way are shown in Fig. 1.

During the course of an experiment the muscle was exposed to either ACh or carbachol at intervals of from 7 to 10 min. The response to a given concentration of agonist gradually fell; if the rate of decline was greater than a few per cent per hour, an allowance was made by expressing other depolarizations as fractions of the interpolated response to the test dose at the appropriate time. Depolarizations greater than 40–60% of the maximum value evoked by a large concentration of agonist appeared to quicken the normal slow decline in response, and were avoided. It was found important to make a frequent check of the potential difference occurring along the muscle in the absence of any depolarizing agent. This 'injury potential' was occasionally observed to change spontaneously, and quite suddenly, by as much as 1 mV.

It has recently been shown (Gray, 1958) that in the m. ext. long. dig. IV of the frog from 6 to 20% of those fibres of diameter greater than 10μ have the 'Felderstruktur' taken by Krüger (1952) to be characteristic of slow fibres. However, this should not complicate the use of the muscle in the present experiments since these fibres, having a distributed innervation, will be fairly uniformly depolarized by ACh applied over their whole length. They should thus contribute little to potential gradients along the muscle resulting from depolarization of the discretely localized end-plates of the twitch fibres.



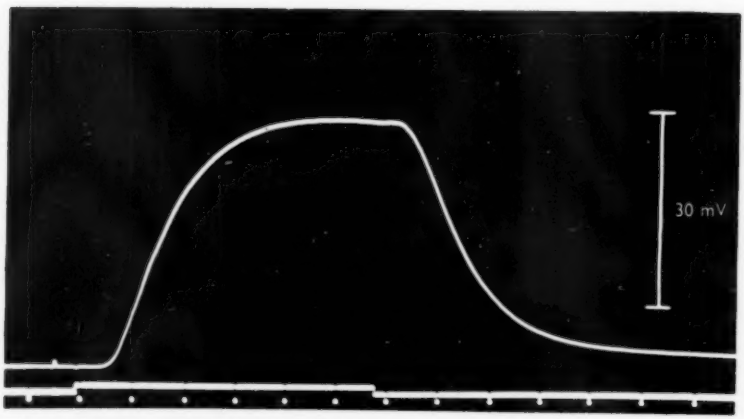


Fig. 1. Upper records: the potential developed (at time of maximal response) along the m. ext. long. dig. IV of the frog on the application of solutions containing (1) 2·9, (2) 2·3, (3) $5{\cdot}8 \times 10^{-6}$ M-carbachol. (4) shows the small 'injury potential' observed in the absence of any depolarizing agent. Extracellular recording. Abscissa, distance along muscle (cm).

Lower record: the action of ACh ($5{\cdot}8 \times 10^{-5}$ M, in the presence of $4{\cdot}7 \times 10^{-6}$ M tubocurarine) on the membrane potential at the end-plate region of a superficial fibre of the frog's sartorius. Resting potential of fibre 93 mV. Time marker 5 sec. The electrical threshold of the fibre has been raised by including twice the normal concentration of Ca, and 4 mM-Mg, in the bathing fluid. Prostigmine 10^{-6} g/ml. present throughout.

Procedure with micro-electrodes

The responses of single end-plates in the sartorius were recorded as described by Fatt & Katz (1951). The muscle lay in a small rectangular groove just covered by a continuously flowing stream of fluid, the composition of which could be changed within a few seconds by means of a six-way tap (similar to that described by Hodgkin & Horowicz, 1959), mounted in direct contact with the bath. Movement artifacts were reduced by clamping the assembly of bath and tap to the base of the micro-manipulator. With this arrangement it was possible to keep a micro-electrode in a fibre for over an hour, during which period the composition of the fluid could be changed many times.

A record obtained in this way is shown in the lower part of Fig. 1. It was noted that the responses to ACh and carbachol, and the action of tubocurarine, developed with approximately exponential time courses, which did not depend on the rate of flow unless this was very small. For a given substance, the half-times to maximum response varied greatly from end-plate to end-plate, e.g. with ACh values of 2–15 sec were observed. It was noted in three experiments that the action of tubocurarine developed at about half the rate of that of ACh, as would be expected if the time courses were determined by diffusion (del Castillo & Katz, 1957).

Solutions

The Ringer's solution had the following composition (mM): Na, 117; K, 2·1; Ca, 1·8; Cl, 121·0; phosphates, 2, to give pH 7. Acetylcholine chloride (Roche) was made up freshly for each experiment and was always used in the presence of neostigmine methyl sulphate (Prostigmine, Roche) 10^{-6} g/ml. At least an hour was allowed for the development of the action of the latter substance. Carbaminoyl choline chloride (Carbachol) and tubocurarine chloride were obtained from Burroughs Wellcome and Co. Dimethyl tubocurarine was supplied by Allen and Hanburys, Ltd.

THEORY

If the antagonism is competitive, either agonist or antagonist may combine with the receptor sites (R). The relationship between the number of sites occupied by the agonist and the depolarization which results is not known with any certainty. However, it may be shown that the following expression should hold if two concentrations, [A] and [A]′, of agonist give the same response in the presence and absence respectively of a concentration [I] of the antagonist:

$$\frac{[A]}{[A]'} - 1 = K[I], \qquad (1)$$

where K is the affinity constant of the reaction $R + I \rightleftharpoons RI$ (Gaddum, 1943). This relationship is based on the assumptions that only one molecule may combine with each site, and that equal responses correspond to equal numbers of sites occupied by the agonist. It will be shown that equation (1) describes the experimental results over a large range of concentrations.

Most, if not all, depolarizing and blocking agents are cations at physiological pH. This suggests that the receptor sites are anionic, and the possibility exists that they may possess some affinity for one or more of the

inorganic cations included in the bathing fluids. If this is the case, a modified form of equation (1) would be expected to apply.

In the presence of one such cation, M, assumed to be at a fixed concentration, [M], throughout, three equilibria must now be considered:

$$A + R \leftrightharpoons RA \quad \text{(affinity constant } K_a\text{)},$$
$$I + R \leftrightharpoons RI \quad \text{(affinity constant } K\text{)},$$
$$nM + R \leftrightharpoons RM_n \quad \text{(affinity constant } K_m\text{)},$$

where A is the agonist, and n the number of molecules of the inorganic ion which may combine with each site R. (RM_n, like RI, is taken to be inactive.)

Applying the law of mass action, it is found that

$$\frac{[RA]}{[R]_0} = \frac{K_a[A]}{1 + K_a[A] + K[I] + K_m[M]^n}, \tag{2}$$

where $[R]_0$ is the total number of receptors. Again assuming that equal responses correspond to equal numbers of sites occupied by agonist, the depolarization equivalent to [RA] will be elicited, in the absence of antagonist, by a concentration $[A]'$ of agonist where

$$\frac{[RA]}{[R]_0} = \frac{K_a[A]'}{1 + K_a[A]' + K_m[M]^n}. \tag{3}$$

From (2) and (3) it follows that the expression equivalent to equation (1) is

$$\frac{[A]}{[A]'} - 1 = \frac{K[I]}{1 + K_m[M]^n}. \tag{4}$$

Equation (4) can only be applied when the identity of M, and its affinity for the receptor, are known; in the present work, 'affinity constants' have been calculated by using equation (1). It should be noted that the values of K so derived correspond to the true affinity constant only if $K_m[M]^n \ll 1$. If on the other hand, $K_m[M]^n \gg 1$, so that a negligible number of sites are left unoccupied, equation (4) approximates to

$$\frac{[A]}{[A]'} - 1 = K' \frac{[I]}{[M]^n},$$

where K' $(= K/K_m)$ is the equilibrium constant of the reaction $I + RM_n \rightleftharpoons RI + nM$. The combination of the drug with its receptor would then be described as an 'ion exchange reaction', as first suggested by Ing & Wright (1932).

RESULTS

The first experiments were made with the m. ext. long. dig. IV of the frog. The aim was to obtain a measure of the effect of tubocurarine on the response to depolarizing agents. The muscle was exposed to a series of solutions containing ACh, or carbachol, and the resulting depolarizations of the end-plate region were measured by using a liquid–air interface as an extracellular electrode (see Methods). Dose–response curves were determined with and without tubocurarine in the bathing fluid. A typical experiment of this kind, summarized in Table 1, is illustrated in Fig. 2.

To determine if equation (1) applies to the antagonism, the measured

TABLE 1. Time course of an experiment to test equation (1). Col. 3 lists depolarizations of the end-plate region of m. ext. long. dig. IV produced by the concentrations of carbachol shown in Col. 2 ([A]). During the first 36 and the last 60 min no tubocurarine was present; from 36 to 96 min 2×10^{-6} M, and from 96 to 160 min 4×10^{-6} M, tubocurarine was included in all solutions. To allow for the slow decline in end-plate response with time, depolarizations have been expressed (Col. 4) as a fraction of the interpolated response to an arbitrary standard dose ($1{\cdot}08 \times 10^{-5}$ M) of carbachol, as described in Methods. The values listed in Col. 6 were calculated from the ratios of [A] to [A]′ by using equation (1) (see text)

Times (min)	[A] ($\times 10^{-6}$ M)	Depolarization (mV)	Responses, as fraction of that to $1{\cdot}08 \times 10^{-5}$ M carbachol	[A]′ ($\times 10^{-6}$ M)	K ($\times 10^{6}$ M^{-1})
0	7·2	5·45	0·615	—	—
7	3·6	1·9	0·22	—	—
15	9·0	7·1	0·82	—	—
25	5·4	3·55	0·415	—	—
34	10·8	8·45	1·00	—	—
62	42·6	5·7	0·71	8·2	2·10
71	54·0	6·75	0·845	9·45	2·35
83	24·8	2·25	0·29	4·3	2·45
92	33·5	3·05	0·47	6·0	2·30
120	91·5	5·9	0·78	8·85	2·30
130	40·5	1·8	0·25	3·90	2·35
141	71·5	3·9	0·54	6·65	2·40
158	53·9	2·65	0·38	5·1	2·40
200	10·8	6·5	1·00	—	—
210	4·5	1·9	0·30	—	—

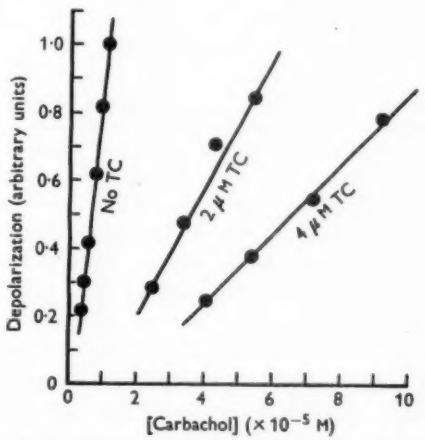


Fig. 2. Plot data listed in Table 1. Two of the lines, those referring to depolarizations measured in the presence of antagonist, have been constructed from the remaining one by introducing the best value of K ($2{\cdot}4 \times 10^{6}$ M^{-1}; see legend to Table 1) into equation (1).

depolarizations were plotted against the concentration of agonist, as in Fig. 2, and a line (which was often, as in this particular case, straight) was drawn through those points obtained in the absence of tubocurarine. This was used to determine for every other point the concentration [A]′ of agonist which would have given the same response had there been no antagonist present. Equation (1) could then be tested by using it to calculate a value for K, the affinity constant for tubocurarine, from the ratio [A]/[A]′. The values obtained should be the same for each point. It may be seen from Col. 6, Table 1 that this was so for the experiment of Fig. 2.

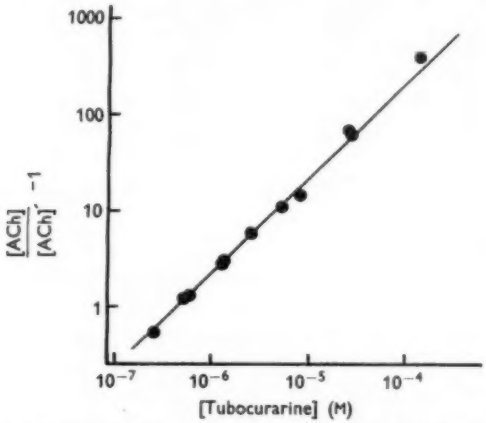


Fig. 3. Results of a series of experiments to test the applicability of equation (1) over a range of antagonist concentrations. Procedures as described in text. No more than two points determined with any one muscle. ACh as agonist; prostigmine 10^{-6} g/ml. included in all bathing fluids. Log. scales.

A series of such experiments were made to test equation (1) over a wider range of antagonist concentrations. As before, dose–depolarization relationships were determined with and without tubocurarine in the bathing fluid. In each experiment, the ratio [A]/[A]′ was calculated (for a given concentration, [I], of the antagonist) at three levels of end-plate response; generally depolarizations of about 10, 20 and 40 % of the maximum value were chosen. The three values of [A]/[A]′ were averaged (see next paragraph) and the mean, less unity, is shown plotted against [I] in Fig. 3. The line has been drawn according to equation (1), with K equal to $2{\cdot}20 \times 10^{6}$ M^{-1}, this being the mean value obtained in six experiments in each of which the same concentration of tubocurarine ($1{\cdot}27 \times 10^{-6}$ M) was used. The good agreement between the theoretical line and points measured at other concentrations shows that equation (1) holds over a several hundredfold range of values of [I]. There is some deviation with more than

5×10^{-5} M tubocurarine. This is probably related to the finding that the drug in such large concentrations appeared to quicken the normal slow decline in response.

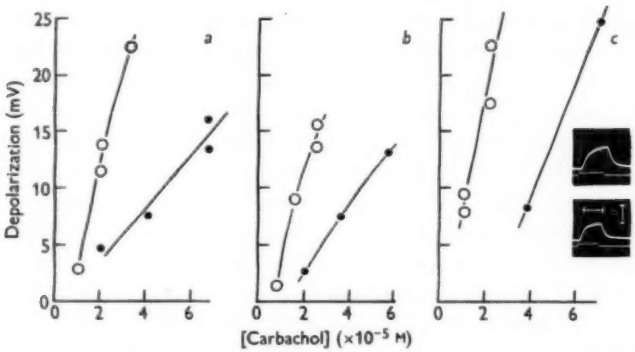
If the assumptions on which equation (1) is based are correct, experimentally determined values of the ratio [A]/[A]′, and so of K, should be independent of the level of end-plate depolarization at which the measurements were made. This point was checked by a further analysis of the experiments described in the preceding paragraph. For each [I], two values of K were calculated from the [A]/[A]′ ratios measured for depolarizations which were approximately 10 and 40% of the maximum value obtainable with a large dose of agonist. The means of these values were found to be $2{\cdot}09 \pm 0{\cdot}09$ (S.E. of mean) and $2{\cdot}23 \pm 0{\cdot}09 \times 10^6$ M^{-1} respectively (10 experiments). These agree sufficiently well to justify averaging [A]/[A]′ ratios obtained at several different levels of response of a given muscle.

A further test of equation (1) was made by comparing affinity constants measured using (*a*) ACh and (*b*) carbachol as a depolarizing agent. Since only a ratio of agonist concentrations need be known, the values should be similar. In four experiments with carbachol, the affinity constant for tubocurarine was found to be $2{\cdot}40 \pm 0{\cdot}03 \times 10^6$ M^{-1}, and with ACh, in the presence of 10^{-6} g/ml. prostigmine, $2{\cdot}20 \pm 0{\cdot}05 \times 10^6$ M^{-1} (six experiments). In three control experiments with carbachol, also with 10^{-6} g/ml. prostigmine, a value of $2{\cdot}3 \pm 0{\cdot}1 \times 10^6$ M^{-1} was obtained. The good agreement between these figures provides additional evidence that equation (1) may be applied to the action of tubocurarine.

Study of the antagonism at single end-plates

The experiments described in the previous section showed that a reproducible index (K, equation (1)) of the action of tubocurarine could be determined through the use of electrical recording methods. However, extracellular recording from a whole muscle can provide only limited information about the responses of individual end-plates. If these were to differ in their affinity for tubocurarine, the 'affinity constant' determined by using this technique would represent a mean value for the fibre population. To find whether the end-plates do vary in this way, a series of experiments was made in which the antagonism between carbachol and tubocurarine was studied by recording the responses of single fibres in the frog's sartorius (see Methods section). As in previous experiments, similar depolarizations were evoked in the presence and absence of a known concentration of tubocurarine. In all cases Mg was added to the bathing fluid, or the concentration of Ca increased, in order to raise the electrical threshold of the muscle fibres. This was to prevent twitching on the application of comparatively large concentrations of carbachol. Three such

experiments are illustrated in Figs. 4*a*, *b* and *c*. As before, affinity constants were calculated from equation (1). Values obtained in eight experiments of this kind are listed in Table 2. It can be seen that K does not vary greatly from end-plate to end-plate, and that the mean value, $1{\cdot}37 \times 10^6$ M^{-1}, agrees well with that obtained on recording externally from the whole m. ext. long. dig. IV (Table 4, rows 3 and 4).



Figs. 4*a*, *b* and *c*. Determination of affinity for tubocurarine of three separate end-plates. Micro-electrodes were used to measure the depolarizations produced by different concentrations of carbachol applied with (●) and without (○) $1{\cdot}27 \times 10^{-6}$ M tubocurarine in the bathing fluid. The resting potential of the fibre of Expt. *a* was 82 mV at the beginning, and 76 mV at the end. The corresponding figures for Expts. *b* and *c* were 82 and 78, and 94 and 90 mV, respectively. To raise the threshold, 3× the normal concentration of Ca was included in the Ringer's fluid of Expt. *a*, and 8·6 mM-Mg in that of Expts. *b* and *c*. The values obtained for K are included in Table 2.

Inset to Fig. 4*c*; records of depolarizations produced by $2{\cdot}2 \times 10^{-5}$ M carbachol at the beginning and at end of Expt. *c*; this end-plate responded particularly slowly to the depolarizing agent. Voltage and time calibrations 20 mV and 1 min, respectively.

TABLE 2. Affinity constants ($\times 10^6$ M^{-1}) for tubocurarine determined by recording the responses of single end-plates. Bathing fluid contained 5·4 mM-Ca in the experiments of Col. 1, and 8·6 mM-Mg in those of Col. 2

	1	2
	1·4	1·1 (Fig. 4*b*)
	1·2 (Fig. 4*a*)	1·7 (Fig. 4*c*)
	1·4	1·6
	1·0	1·6
Mean	1·25	1·5

Dose–depolarization relationship

It was observed (cf. Figs. 2, 4*a*, *b* and *c*) that the dose–response relationship often had a linear portion which, on extrapolation, did not pass

through the origin. Two further examples, one from a single end-plate, are illustrated in Fig. 5. Cavanaugh & Hearon (1954) and Kirschner & Stone (1951) have reported a similar finding for contracture–dose relationships of the frog's rectus.

Slow fibre receptors

In contrast to 'twitch' muscles such as the sartorius, the rectus responds to the bath application of depolarizing agents with a well maintained

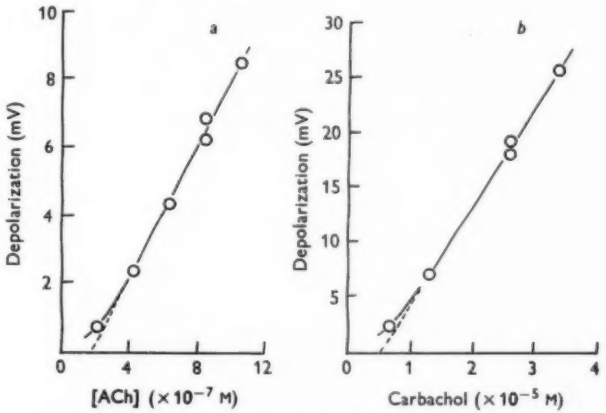


Fig. 5. Initial portions of dose–depolarization relationships, determined by recording the responses of (*a*) the m. ext. long. dig. IV, with extracellular electrodes, and (*b*) a single end-plate of the frog's sartorius (resting potential of fibre 93 mV). Prostigmine 10^{-6} g/ml. present throughout experiment of Fig. 5*a*. It may be seen (dotted lines) that the linear regions of the curves, if extrapolated, do not pass through the origin.

TABLE 3. Affinity constants ($\times 10^6$ M^{-1}) for tubocurarine determined by taking as a measure of drug action (*a*) contractures of the frog's rectus, (*b*) depolarizations of the m. ext. long. dig. IV, measured with external electrodes (values taken from Table 4). Mean values, S.E. of the mean, and number of experiments are listed

Bathing fluid	*a*	*b*
Ringer's solution	3·15 ± 0·05 (3)	2·30 ± 0·05 (13)
Ringer's solution + 8·6 mM-Mg	1·66 ± 0·07 (4)	1·40 ± 0·07 (7)

contracture, known to originate from the 'slow' fibres (Kuffler & Vaughan Williams, 1953). If this response is used as a measure of drug action, any conclusions about affinities will hold only for the receptors of these slow fibres, which may well differ from those of the 'twitch' type. To enable a comparison of the affinities of the two receptors to be made, experiments were carried out in which contracture–dose relationships of the rectus were

determined with and without tubocurarine in the bathing solution. As before, affinity constants were calculated from equation (1). Values obtained in seven such experiments are shown in Table 3, together with others determined by recording externally from the m. ext. long. dig. IV, a predominantly 'twitch' muscle. It may be seen that the affinities for tubocurarine of the two fibre types are quite similar.

The effect of changes in temperature, and in the Ringer's fluid, on the affinity for tubocurarine

In an attempt to obtain more information about the mechanism of the tubocurarine–receptor reaction, affinities were determined under other experimental conditions. It may be seen from Fig. 6 that there is little dependence on temperature over the range 3–30° C, Q_{10} probably lying

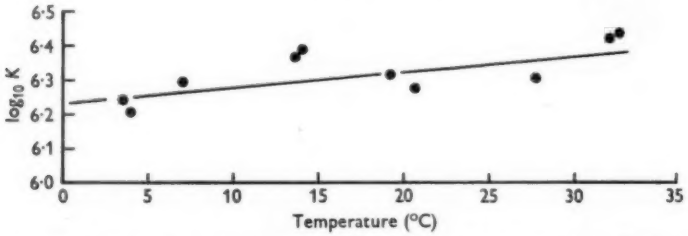


Fig. 6. The effect of temperature on the affinity for tubocurarine. Ordinate: $\log_{10} K$ (M^{-1}); abscissa, temperature. Values determined by extracellular recording from the m. ext. long. dig. IV of the frog.

TABLE 4. Affinity constants for tubocurarine determined in modified Ringer's solutions. Values determined by recording externally from the m. ext. long. dig. IV. Tonicity of low-sodium solutions maintained with sucrose, and of others by the replacement of sodium

Row	Bathing fluid, Ringer's solution	K ($\times 10^6 M^{-1}$)
1	No addition	2·3 ± 0·05 (13 expts.)
2	+6·3 mM-K	2·45, 2·5, 2·25
3	+5·4 mM-Ca	1·8, 1·5, 1·7
4	+8·6 mM-Mg	1·6, 1·5, 1·5, 1·3, 1·5, 1·2, 1·3
5	Containing 2/3 [Na]	2·5, 2·6
6	Containing 1/3 [Na]	5·8, 5·3

between the limits 0·95 and 1·10. Van Maanen (1950), recording contractures of the frog's rectus, also concluded that the affinity for tubocurarine was almost, if not completely, independent of temperature.

The effect of changes in the concentration of several cations is shown in Table 4. It may be seen that the replacement of sodium by sucrose results in an increase in the affinity constant (cf. the 'curarizing' action of low-sodium solutions, Fatt & Katz, 1952). The addition of magnesium, and an

increase in the concentration of calcium, have the opposite effect. A four-fold increase in the potassium concentration has little action, as found by van Maanen (1950) for the frog's rectus.

Tubocurarine and denervated muscle

It is well known that the sensitivity of a muscle to depolarizing agents becomes greater on denervation. A number of experiments were made to find whether the affinity for tubocurarine also increases. The lower portion of one hind limb of a frog was denervated by removal of about 1 cm of the sciatic nerve. At least 3 weeks later, the affinity constant of the m. ext. long. dig. IV of that limb was determined in an experiment of the kind illustrated in Fig. 7. The results of this and of eight other such

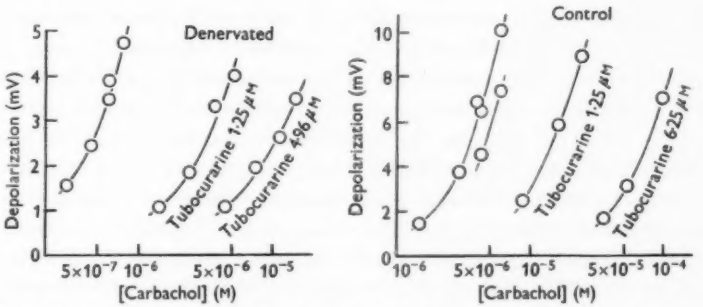


Fig. 7. Comparison of affinities for tubocurarine of normal and denervated (32 days) muscles from the same frog. Procedure as described in legend to Table 1. Values obtained have been included in Table 5. Semi-log scale.

TABLE 5. The effect of denervation on the affinity for tubocurarine (Expts. 1–8). Cols. 3 and 4 list the affinity constants (K_D and K_N respectively) of the denervated and, in some cases, of the unoperated muscles from the same animal. Cols. 5 and 6 show the ratios of the doses of ACh ($+10^{-6}$ g/ml. prostigmine) and carbachol which produce the same depolarization in the control and denervated muscle, respectively. All values determined by recording extracellularly from the m. ext. long. dig. IV of the frog. One experiment with dimethyltubocurarine, No. 9, has been included

1	2	3	4	5	6	7
Expt.	Time denervated, days	K_D ($\times 10^6$ M^{-1})	K_N ($\times 10^6$ M^{-1})	R_{ACh}	R_{carb}	K_D/K_N
1	23	2·95	2·45	6	—	1·2
2	30	3·45	2·20	15	—	1·6
3	31	3·40	—	10	—	—
4	34	3·40	—	10	5	—
5	35	3·20	2·40	11	5	1·3
6	37	3·00	2·40	16	5	1·25
7 (Fig. 7)	32	4·60	2·40	25	5	1·9
8	64	3·40	—	85	—	—
9	69	2·00	1·40	30	9	1·4

determinations are listed in Table 5. It can be seen that the affinities for tubocurarine of the denervated muscles are larger than those of the controls, but that the increase in the sensitivity to ACh and carbachol is much greater.

A puzzling feature of the present experiments was that the supersensitivity (cols. 5 and 6, Table 5) of the denervated muscles never became as great as that described by Nicholls (1956) (sensitivity to ACh, with prostigmine 10^{-6} g/ml., increased by a factor of from 50 to 100) or by Kuffler (1943) (denervated muscles at least a thousand times more sensitive to ACh). No explanation for this variation can be given as yet (cf. Miledi, 1960).

DISCUSSION

The present results provide additional support for the hypothesis that tubocurarine acts by competing with depolarizing agents for some site or receptor molecule. The main finding is that equation (1), derived on the basis of competitive inhibition, is followed over a large range of concentrations (Fig. 3). This may be discussed in relation to a recent suggestion (Shanes, 1958) that neuromuscular blocking agents such as tubocurarine may act by stabilizing the membrane against permeability changes through becoming associated with sites or receptors quite distinct from those with which the agonist combines, so that the antagonism can be described as uncompetitive. This hypothesis is compatible with the present results only if it is assumed in addition that a maximal response can be evoked through the occupation of a very small fraction of the available agonist sites. This follows from a consideration of the range of concentrations over which equation (1) holds. It has been found (Fig. 3) that a given submaximal response may be produced by a sufficiently large dose of agonist even in the presence of more than 10^{-4} M tubocurarine, and then can be even further increased by additional agonist, suggesting that there are still vacant sites. If agonist and antagonist combine with separate receptors, as proposed by Shanes, only a minute fraction of the available agonist sites can be occupied when a similar response is evoked in the absence of tubocurarine by less than 1 % of the previous agonist concentration. The existence of such a large reserve of sites seems unlikely, but of course has not been excluded.

Desensitization. As a rule, the response of the end-plates to depolarizing agents is not well maintained. There is evidence to suggest that this is due to a change, to an inactive form, in the receptors themselves (Katz & Thesleff, 1957). This 'desensitization' would complicate the study of the antagonism if the presence of an appreciable number of modified receptors was to affect the properties of the remainder. However, this did not seem

to have been a source of error under the conditions of the present experiments. This is inferred from a comparison of affinity constants determined by recording from single end-plates and from whole muscles. With the former technique, the observed depolarizations were generally complete in less than 40 sec, whereas responses recorded extracellularly from the m. ext. long. dig. IV reached their maximum only after from 1 to 4 min, at which time the superficial fibres may have been appreciably desensitized. If this were to have introduced a systematic error, affinity constants measured by the two techniques would be expected to differ. However, the values obtained were in fair agreement.

Dose-depolarization curves. These were observed to have an initial region of increasing slope. Such dose–effect relationships have been found with many drugs and tissues, and may arise in several ways, e.g. if each receptor combines with more than one molecule of agonist (as suggested by Cavanaugh & Hearon, 1954, for the action of ACh on the frog's rectus muscle). However, the present results do not favour this explanation, for the antagonism is best described as a competition for sites which are univalent for both agonist and antagonist.

Gaddum (1937) has pointed out that S-shaped curves will occur if the effect measured is the net response of a number of elements of differing sensitivities. The fact that such dose–effect curves are observed on recording the responses of single end-plates, and even on applying carbachol iontophoretically to subunits of end-plates (Katz & Thesleff, 1957), suggests that if an explanation of this type holds, the units of varying sensitivities are small groups of receptors, or possibly the receptors themselves.

Effect of inorganic cations. Magnesium and calcium appeared to reduce the affinity of the receptor for tubocurarine, and the replacement of sodium by sucrose to increase it. These actions would be expected if the receptors have a tendency to combine with these ions (p. 312). However, in the case of sodium, there is a second possibility, namely, that the apparent change in affinity is a consequence of the reduction in ionic strength. It may be estimated from the Debye–Hückel–Onsager theory of electrolytes that if the receptor were to bear a charge equal and opposite to that of the tubocurarine cation, the affinity constant would be about twice as large in a solution containing a third of the normal sodium concentration, as a consequence of the effect of the reduction in ionic strength on the activity coefficients of the ions taking part in the equilibrium. Although the curarization observed was of this order (Table 4), a quantitative allowance for changes in ionic strength cannot be made with certainty until more is known of the receptor, and of the charge borne by it.

The actions of calcium and magnesium, on the other hand, are too large to be accounted for as a consequence of the change in ionic strength

alone. As would be expected if it were to compete for the receptor, magnesium (unlike calcium) decreases the depolarization evoked by bath-applied ACh (del Castillo & Engbaek, 1954). However, del Castillo & Engbaek found that if this experiment was repeated in the presence of tubocurarine, magnesium then *increased* the response to ACh, whereas a decrease, as before, would be expected on an 'ion exchange' theory of the drug–receptor reaction.

A fourfold increase in the concentration of potassium had little effect. In summary, although sodium, calcium and magnesium appear to reduce the affinity constant for tubocurarine, the evidence is not sufficiently clear-cut for it to be concluded that these ions 'decurarize' through a tendency to combine with the receptors.

Denervated muscle. Table 5 shows that the affinity for tubocurarine increases on denervation. This may reflect some change in the receptors; however, the increase in sensitivity to ACh and carbachol is much larger. These findings are consistent with recent evidence that the supersensitivity of denervated muscles to depolarizing agents results from an increase in the receptor area, rather than from a *marked* change in the receptors themselves (Axelsson & Thesleff, 1959; Miledi, 1960).

Slow fibre receptors. Van Maanen (1950) has shown that changes in temperature, and in the potassium concentration, have little effect on the affinity of the 'slow' fibres for tubocurarine. In these respects twitch fibres are similar (Fig. 6, Table 4). Also, the affinity constants for tubocurarine, and the magnitude of the 'decurarizing' action of magnesium, are of the same order (Table 3). These findings suggest that the receptors of the two fibre types, if not identical, resemble each other closely, even though the end-plates in which they occur have marked differences in function and structure.

SUMMARY

1. A quantitative study of the antagonism between ACh and tubocurarine has been made by taking depolarizations of the end-plate region of skeletal muscle as a measure of drug action.

2. The results are consistent with the hypothesis that these substances compete on a one-to-one basis for receptors at the end-plate.

3. Micro-electrodes were employed to show that individual end-plates do not vary greatly in their sensitivity to tubocurarine, as measured by the 'affinity constant' K (equation (1)).

4. K was found to be about $2{\cdot}3 \times 10^6 \text{M}^{-1}$ and was little affected by changes in temperature (3–30° C) or by a fourfold increase in the potassium concentration. Values determined in the presence of 7·2 mM calcium and 8·6 mM magnesium were smaller by about 30 and 40 % respectively;

replacement of 2/3 of the normal sodium by sucrose increased K by from two to three times.

5. In a few experiments affinity constants, presumably of the slow-fibre receptors, were determined by measuring contracture responses of the frog's rectus. Similar values were obtained.

6. The affinity for tubocurarine increased on denervation but not to the same extent as the sensitivity to ACh and carbachol.

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A COMPARISON OF THE UPTAKES OF OXYGEN-15 AND OXYGEN-16 IN THE LUNG

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Oxygen-15 is a positron-emitting isotope with a half-life of 2 min, and has been prepared by a continuous flow method in the Medical Research Council's cyclotron at Hammersmith Hospital. Its properties make it particularly suitable for the investigation of pulmonary physiology in man. Regional lung function in normal subjects and in patients with pulmonary disease has been assessed by external counting of radioactivity in defined regions of the lungs following inspiration of air labelled with this radioisotope (Dyson, Hugh-Jones, Newbery, Sinclair & West, 1960). A single breath of the labelled air is taken, and is held for 10–15 sec. Two pairs of scintillation counters placed in front of and behind the chest detect the initial rise of the activity in the lung between them and its subsequent fall during the breath-holding period. The initial activity is a measure of the ventilation of the defined volume, and the rate of fall during breath-holding (defined as the 'oxygen-15 clearance rate') is determined chiefly by the regional blood flow.

It has been found that the rate of uptake of $^{15}O_2$ into the blood is greater than that of the naturally occurring $^{16}O_2$. This paper describes some work designed to measure the ratio of clearance rates of $^{15}O_2$ to $^{16}O_2$ under various conditions, and shows how the experimental results can be reconciled with a theoretical approach. (The number of ^{15}O atoms is a very small proportion of the total (see below). This, together with a consideration of the method of production, indicates that the radioactive molecules will be $^{15}O^{16}O$, not $^{15}O_2$. However, the latter form is used for convenience and brevity.)

METHODS

Oxygen-15 is prepared in the cyclotron by the bombardment of nitrogen with the external deuteron beam at an energy reduced to 3 MeV. Air from a cylinder is slowly passed through a box into which the deuteron beam is projected and a very small proportion of the nitrogen is converted to $^{15}O_2$ by the (d, n) reaction. Radiochemical and other impurities are removed

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as described by Dyson, Hugh-Jones, Newbery & West (1958), and the gas is conducted to an adjacent investigation room where its activity is between 50 and 100 mc/l. For the experiments to be described this gas was diluted about twentyfold, either with air, nitrogen or oxygen: the proportions of oxygen in the resulting mixtures were 21, 1 or 96 %, respectively (of which only one part in 10^{11} was $^{15}O_2$). The radioactivity of the mixtures inhaled was about 3 mc/l. The absorbed dose of radiation per breath has been calculated and is in the region of 5 mr to the gonads, 20 mr to the blood-forming tissue and 100 mr to the lungs. Although these doses are small, the number of breaths was kept to the minimum necessary for obtaining useful results.

The activity of the diluted gas was measured by passing it through a vessel in a re-entrant ionization chamber, and then via a three-way tap to waste. By turning the tap, a 5 l. bag could be filled; this was connected by another three-way tap to a simple respiratory valve box. The subject sat in a chair, and breathed room air through the valve box. For the test he was connected to the bag after a full expiration, and inhaled the radioactive mixture to the level of full inspiration. He then quickly exhaled about 2 l. of gas, the volume being recorded on a spirometer, and a sample of this expirate was taken in an evacuated tonometer placed just beyond the expiratory valve. The subject held his breath for 15 sec, and then exhaled fully. A sample of this second expirate was collected in another tonometer.

The experiments were carried out on normal male staff aged 28–35 years. All three gas mixtures were given to resting subjects previously breathing room air, the high-oxygen mixture to three subjects previously breathing 100 % O_2 for 5 min, and the mixture in air to one subject after 3 min of fairly strenuous exercise. For this the subject pushed foot pedals against resistant springs at a rate which increased the oxygen uptake to 1100–1200 ml./min. The labelled air was given immediately after the exercise.

The radioactivity of the $^{15}O_2$ in the tonometer was measured in a re-entrant ionization chamber, by using a vibrating reed electrometer to measure the current. The difference between the two samples divided by the activity in the first sample gave the loss of $^{15}O_2$ relative to the amount originally present. A correction had to be made for radioactive decay.

In general, each member of a pair of tonometers differed slightly in volume and a correction was therefore necessary. A calibration procedure was used in which the two tonometers were connected in series, with air labelled with $^{15}O_2$ of fixed activity per unit volume passing through them. Any difference between ionization-chamber readings of the two tonometers filled in this way must be due to a difference in volume, or to a slight difference in shape which could affect the response of the ionization chamber. The correction factor was thus determined directly.

In each determination a large number of readings of both tonometers were taken, and in about half the determinations the standard error was calculated. It never exceeded 1 % of the initial activity. Any systematic error introduced by error in the correction term would normally be less than this.

After the activity in the tonometers had been measured, the $^{16}O_2$ concentration was determined. The gas was gradually displaced by using a mercury levelling bulb and the sampling tube of the respiratory mass spectrometer was placed in the gas stream. The $^{16}O_2$ concentration could be followed on a pen recorder. About six readings were made on each tonometer and duplicate agreement was better than 1 % pro rata. The spectrometer tends to read high in mixtures containing large concentrations of oxygen. However, when comparing two tonometer samples containing similar O_2 concentrations, differences in concentration of 1 % pro rata are measurable. The clearance rate was calculated in the same way as for $^{15}O_2$.

It is concluded that the accuracy of the $^{15}O_2$ and $^{16}O_2$ determination was such that the calculated clearance rates are accurate to better than 2 % of the initial values.

RESULTS

Results are shown in detail in Table 1 and in summary in Table 2. After breathing $^{15}O_2$ in air the alveolar partial pressure of $^{15}O_2$ in five resting subjects fell by a mean of 50 %, while that of $^{16}O_2$ fell by 20 %. The ratio

TABLE 1. Percentage fall in $^{15}O_2$ and $^{16}O_2$, and ratio of these for each breath

	Fall in $^{15}O_2$ (%)	Fall in $^{16}O_2$ (%)	Fall in $^{15}O_2$ / Fall in $^{16}O_2$
$^{15}O_2$ in air at rest	48	18	2·7
	51	14	3·7
	68	25	2·7
	*51	*22	*2·4
	53	20	2·7
	51	26	2·0
	43	14	3·1
	52	21	2·5
Means	50	20	2·5
$^{15}O_2$ in air after exercise	60	46	1·3
	63	53	1·2
Means	62	50	1·2
$^{15}O_2$ in nitrogen	64	4	16
	54	15	3·6
	59	−1·5	−39
	63	18	3·5
Means	60	9	6·7
$^{15}O_2$ in O_2 (single breath)	20	12	1·7
	24	15	1·6
	22	10	2·2
	28	13	2·2
Means	24	13	1·8
$^{15}O_2$ in O_2 (steady state)	18	1·4	13
	20	3·0	6·7
	9	5·2	1·7
Means	16	3·2	5·0

* First four entries refer to preliminary experiments which were not timed accurately; they are therefore excluded from the means.

TABLE 2. Mean values for the five groups of experiments

	$^{15}O_2$ in				
	Air (rest)	Air (exercise)	N_2	O_2*	O_2†
Number of subjects	5	1	3	3	3
Total number of breaths	8	2	4	4	3
$P_{A_{O_2}}$ (onset of breath-holding, mm Hg)	131	122	48	464	674
Fall in $^{16}O_2$ (%)	20	50	9	13	3
Fall in $^{15}O_2$ (%)	50	62	60	24	16
Fall in $^{15}O_2$ / Fall in $^{16}O_2$	2·5	1·2	6·7	1·8	5·0

* Oxygen given as a single breath.
† Oxygen given under steady state conditions.

of the clearances of the two isotopes varied in eight test breaths from 2·0:1 to 3·7:1, the mean, excluding the preliminary experiments, being 2·5:1. Measurements were made in one subject just after exercise, giving a ratio of 1·2:1. This was because there was a great increase in $^{16}O_2$ uptake but a small increase in $^{15}O_2$ uptake.

When the labelled air was diluted with nitrogen the conditions of the experiments produced alveolar O_2 tensions of the order of those of mixed venous blood. In one instance the sample after breath-holding contained $^{16}O_2$ at a higher partial pressure than that before, indicating that $^{16}O_2$ was excreted from mixed venous blood during this time. In this experiment the $^{15}O_2$ clearance was 59 %. The difference in the behaviour of the two isotopes is thus especially marked in this instance. In all four breaths with the mixture of low oxygen content, $^{15}O_2$ clearance rates were higher than those which occurred with the air mixture. The ratio of the clearance of $^{15}O_2$ to $^{16}O_2$ varied considerably because in these circumstances $^{16}O_2$ uptake is so sensitive to alveolar oxygen tension.

Administration of $^{15}O_2$ in a mixture of high O_2 content produced different results when given as a single breath from those obtained when high-oxygen mixtures had been inhaled beforehand. In the single breath, alveolar O_2 tension was high with a mean of 464 mm Hg; there was a surprisingly big uptake of $^{16}O_2$ in the 15 sec of breath-holding (279 ml.) and the ratio of clearance of $^{15}O_2$ to $^{16}O_2$ was 1·8:1. In the steady-state procedure, alveolar O_2 tension was higher, the mean value being 674 mm Hg; $^{15}O_2$ clearance was moderately reduced, but the $^{16}O_2$ clearance rate was greatly reduced, and the ratio rose to 5·0:1.

DISCUSSION

Measurements made under a variety of conditions have shown that $^{15}O_2$ uptake, expressed as a fraction of the initial alveolar concentration, is faster than $^{16}O_2$ uptake. This is readily explicable when the dynamic nature of O_2 transfer is appreciated. Molecules of O_2 are continually passing to and fro across the alveolar membrane as the pulmonary blood moves along the capillary. Forward flow of O_2 from gas to blood is proportional to the gas partial pressure, and backward flow to the blood gas tension, so that the net transfer at any moment is proportional to the difference between these. Initially the blood contains no $^{15}O_2$ but an appreciable partial pressure of $^{16}O_2$, so that the fractional uptake for the radioactive isotope is faster, and this difference is maintained along the capillary.

It is possible to study the course of uptakes of the two isotopes along the pulmonary capillary by dividing the time a corpuscle spends in the capillary into many small equal intervals. Bohr (1909) originally used this

technique to determine the time course of $^{16}O_2$ tension and saturation along the capillary. A reasonable value for the diffusion constant is assumed and the volume of O_2 which enters the blood in the first Δt sec is calculated from the initial partial-pressure gradient. The change in partial pressure of O_2 which accompanies this volume change is then found, and this gives the new partial-pressure gradient. Thus the volume which is transferred in the next Δt sec is derived and the process is repeated stepwise along the capillary.

The chief difficulty in the case of $^{15}O_2$ is finding the partial-pressure change for a known volume change—in effect, the $^{15}O_2$ dissociation curve in the presence of $^{16}O_2$. This is simplified, however, because the concentration of $^{15}O_2$ is exceedingly small compared with $^{16}O_2$ so that although the presence of $^{16}O_2$ affects the $^{15}O_2$ dissociation curve, the converse does not occur. If in a mixture of the two isotopes with haemoglobin the chemical behaviour of all oxygen molecules is assumed to be the same, at equilibration the concentrations of 15-oxyhaemoglobin and 16-oxyhaemoglobin (C_{15}, C_{16}) will be proportional to the corresponding gas tensions (P_{15}, P_{16}). Thus $P_{15}/C_{15} = P_{16}/C_{16}$, where C is the concentration of O_2 present (vol. O_2/vol. blood). The dissociation curve for $^{15}O_2$ will therefore be determined by the prevailing $^{16}O_2$ partial pressure and content. The partial pressure of $^{15}O_2$ is at all times proportional to the radioactivity per unit volume of the gas mixture.

An attempt was made to verify these dissociation curves by equilibrating a mixture of $^{15}O_2$ and $^{16}O_2$ with blood in a tonometer. However, the half-time taken for equilibration under these conditions was of the same order as the half-life of the radioactive isotope and this precluded a valid measurement.

By using these dissociation curves the change in partial pressure for the concentration change of $^{15}O_2$ in each Δt sec can be found. Graphs of both partial pressure and concentration against time along the capillary can then be drawn (for any moment during breath-holding) if one assumes a value for the saturation of mixed venous blood. An example of a calculation (using the alveolar partial pressure at the beginning of breath-holding) is given in Appendix I. Figure 2 shows the changes along the capillary for normal subjects at rest with alveolar O_2 partial pressures of 48, 131 and 675 mm Hg and for a normal subject, after exercise, with an alveolar partial pressure of 122 mm Hg. These values correspond to the means for the reported experiments. All $^{15}O_2$ figures have been scaled up for convenience by a constant equal to the initial partial pressure of $^{16}O_2$ over that of $^{15}O_2$.

The diffusion constant used in this analysis is related not only to the roperties of the alveolar membrane but also to those of the red cell and

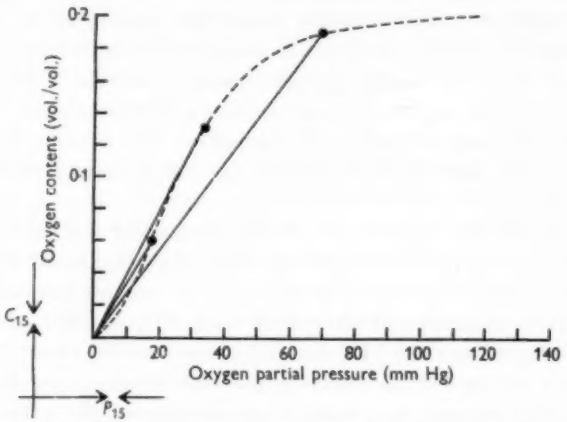

Fig. 1. The relations between the partial pressures and contents of minute concentrations of $^{15}O_2$ in the presence of $^{16}O_2$. The three continuous lines are dissociation curves of $^{15}O_2$ for three prevailing $^{16}O_2$ partial pressures.

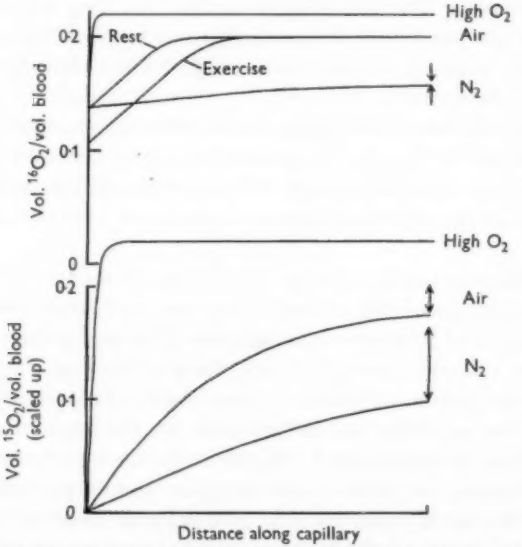

Fig. 2. The calculated rise in the contents of $^{15}O_2$ and $^{16}O_2$ along the pulmonary capillary under various conditions. The subjects were breathing a mixture of high O_2 content (P_{AO_2} = 674 mm Hg), breathing air at rest (P_{AO_2} = 131 mm Hg), breathing air on moderate exercise (P_{AO_2} = 122 mm Hg) and breathing nitrogen P_{AO_2} = 48 mm Hg). The arrows indicate the amount by which the blood O_2 content at the end of the capillary failed to reach the equilibration value. The $^{15}O_2$ curves for the subject breathing air at rest and on exercise were almost identical.

the reaction rates of O_2 with haemoglobin. Roughton & Forster (1957) have shown that only about half of the barrier to the transfer of carbon monoxide from alveolar gas to haemoglobin is due to the alveolar membrane.

It can be seen that the general shape of the curves is similar for the two isotopes, but the rise in concentration of $^{15}O_2$ is slower. As a result, its end-capillary gradient will be larger and the influence of a diffusion defect will be more marked. However, for a subject breathing a mixture of high oxygen content at rest, virtual equilibration of alveolar and blood partial pressures occurs.

In situations where complete equilibration occurs, the concentration of $^{15}O_2$ in end-capillary blood is given by

$$C_{15} = P_{15} \cdot \frac{C_{16}}{P_{16}}.$$

The rate of $^{15}O_2$ uptake is then equal to $P_{15} \cdot C_{16}/P_{16} \cdot \dot{Q}c$, where $\dot{Q}c$ is the cardiac output, and the uptake per unit time expressed as a fraction of total initial alveolar $^{15}O_2$ is

$$P_{15} \cdot \frac{C_{16}}{P_{16}} \cdot \dot{Q}c \frac{1}{V_A \cdot P'_{15} K},$$

where V_A is the alveolar volume, K is a constant and P'_{15} is the initial value of P_{15}. It can be seen that the $^{15}O_2$ concentration will fall approximately linearly with time. If the change in P_{15} during breath-holding is relatively small, the expression becomes simply

$$\frac{C_{16} \cdot \dot{Q}c}{V_A \cdot P_{16} K}. \tag{1}$$

The equivalent expression for $^{16}O_2$ is

$$\frac{\dot{Q}c(C_{16} - Cv_{16})}{V_A \cdot P_{16} \cdot K}, \tag{2}$$

where Cv_{16} is the O_2 concentration in mixed venous blood, whence the ratio of fractional $^{15}O_2$ to $^{16}O_2$ uptakes is constant during the breath-holding period, and is given by

$$\frac{C_{16}}{C_{16} - Cv_{16}}. \tag{3}$$

These expressions assume a constant alveolar O_2 partial pressure. When the slow fall in P_{15} and P_{16} which occurs with breath-holding is taken into account, the expression for the $^{15}O_2$ fractional uptake up to time t from the beginning of breath-holding is

$$1 - (1 - rt)^n, \tag{4}$$

where

$$r = \frac{\dot{Q}c(C_{16} - Cv_{16})}{V_A P'_{16}} \quad \text{and} \quad n = \frac{C_{16}}{C_{16} - Cv_{16}}.$$

It is assumed that C_{16} and Cv_{16} are constant during breath-holding as before; P'_{16} is the initial value of P_{16}.

The ratio R of $^{15}O_2$ to $^{16}O_2$ fractional uptakes up to a given time t from the beginning of breath-holding is now related to t and is given by

$$R = \frac{1-(1-rt)^n}{rt}. \quad (5)$$

The derivation of this expression is given in Appendix (2).

The above expressions assume no alveolar end-capillary gradient. When the subject breathes air or a mixture of low oxygen content, a gradient exists (see Fig. 2) and the $^{15}O_2$ uptake is consequently less. The values of R calculated from equation 5 have been corrected for this effect by using the end-capillary gradients for the beginning of breath-holding. The difference between the end-capillary gradients of the two isotopes will vary during the breath-holding period as the alveolar O_2 partial pressure falls, but this modification to the correction factor has not been taken into account.

It is clear that the factors affecting $^{15}O_2$ uptake are complicated and that any approach by computation must be very approximate. Nevertheless, we have examined the observed results in the light of the foregoing analysis. We have used the results of Donald, Bishop, Cumming & Wade (1955) to derive a figure for cardiac output using the measured values for

TABLE 3. Table showing predicted $^{15}O_2$ clearance rates and principal values (means) on which these are based, together with the observed clearance rates

	Air (rest)	Air (exercise)	N_2	O_2 (single breath)	O_2 (steady state)
Lung volume (l.)	4·2	3·5	4·2	4·2	4·4
$^{16}O_2$ consumption (ml./15 sec)	144	280	24	320	125
Cardiac output (l./min)	10	12·2	10	10	10
Mixed venous saturation (%)	69	53	69	69	82
Observed $^{15}O_2$ clearance (%)	50	62	60	24	16
Predicted $^{15}O_2$ clearance (%)	48	70	49	24	13
Observed $^{15}O_2/^{16}O_2$ clearance	2·5	1·2	6·7	1·8	5·0
Predicted $^{15}O_2/^{16}O_2$ clearance	2·5	1·6	5·5	1·8	4·2

$^{16}O_2$ consumption. No change in this figure was assumed to occur when the subject breathed pure oxygen. Cardiac outputs were generally higher than resting values, presumably because of excitement. The value for mixed venous oxygen saturation following 5 min of breathing O_2 was found by assuming that the volume of O_2 extracted from the arterial blood was the same as for the subject breathing air. However, it can be shown that values obtained from the predictions are insensitive to the mixed venous O_2 saturation.

Table 3 gives the lung volume, $^{16}O_2$ consumption, cardiac output, mixed venous O_2 saturation, observed and predicted $^{15}O_2$ uptake and

ratio of $^{15}O_2$ to $^{16}O_2$ uptake in the five groups of experiments. Agreement between observed and predicted values is reasonable.

There is much less variation in the rate of uptake of $^{15}O_2$ than of $^{16}O_2$ under the different experimental conditions. This is because the $^{16}O_2$ uptake is determined not only by the cardiac output but also by the relation between mixed venous O_2 tension and alveolar O_2 partial pressure, whereas $^{15}O_2$ uptake is virtually independent of the $^{16}O_2$ saturation in mixed venous blood. Thus it is possible for $^{16}O_2$ consumption to decrease considerably when the alveolar oxygen partial pressure falls during a breath of nitrogen, although $^{15}O_2$ uptake is little affected. In these circumstances, the ratio of $^{15}O_2$ to $^{16}O_2$ uptakes becomes very large. Indeed, in one experiment the $^{16}O_2$ partial pressure rose slightly during breath-holding, indicating excretion of this gas, while considerable $^{15}O_2$ uptake was observed. In this experiment, the uptake of $^{15}O_2$ was observed to be greater than when the subject inspired the $^{15}O_2$ in air, and the theoretical analysis predicts such a result.

From the foregoing it may be seen that an abnormally high pulmonary blood flow would be expected to increase the ratio of $^{15}O_2$ to $^{16}O_2$ uptakes. In general, the $^{15}O_2$ uptake will be greater, whereas the $^{16}O_2$ uptake may be unchanged in the resting subject. For example, in a previous paper (Dyson *et al.* 1960) we have reported a high $^{15}O_2$ uptake in a resting subject with a high pulmonary blood flow caused by anomalous venous return. A similar result would be expected in conditions in which the resting cardiac output was abnormally high.

SUMMARY

1. The uptake of the radioactive isotope oxygen-15 has been compared with that of the naturally occurring oxygen-16 under various conditions during a short period of breath-holding in man. Alveolar gas samples exhaled before and after the breath-holding period were analysed for oxygen-15 by using a re-entrant ionization chamber, and for oxygen-16 with a mass spectrometer.

2. Oxygen-15 uptake (expressed as a fraction of the initial concentration) always exceeded oxygen-16 uptake, and in one measurement following a breath of pure nitrogen, oxygen-16 excretion into alveolar gas occurred while oxygen-15 uptake was high.

3. The apparent discrepancy between the uptakes of oxygen-15 and oxygen-16 results from their unequal back pressures in the blood entering the pulmonary capillary. An expression predicting the uptake of oxygen-15 has been obtained and has given reasonable agreement with the observed results.

We should like to thank Dr K. Cotton for useful discussions, Dr P. Hugh-Jones, Mr G. R. Newbery and Professor F. J. W. Roughton for their interest, and Miss Heather MacLeish and Mr P. Buckingham for valuable assistance.

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APPENDIX

(1) As an example of the step-wise calculations along the pulmonary capillary, the first steps for the data for subjects at rest inspiring $^{15}O_2$ in air are set out here. The mean O_2 consumption was 576 ml./min, which corresponds to a cardiac output of 10 l./min (Donald *et al.* 1955). Thus, for an arterial O_2 saturation of 97 % and a blood O_2 capacity of 0·2 vol./vol. the content of mixed venous blood is 0·138 vol./vol. and its saturation 69 %. This corresponds to a tension of 37·5 mm Hg on Riley's physiological dissociation curve (Riley & Cournand, 1949). The mean alveolar O_2 tension was 131 mm Hg.

Now choose a value for the rise in blood O_2 content in the first Δt sec, say 0·01 vol./vol. Since the initial partial pressure gradient equals 131–37·5 = 93·5 mm Hg, the diffusion constant is 0·01/93·5 = 0·000107 vol./vol./mm Hg/Δt sec. The new content is therefore 0·148 vol./vol., corresponding to a new saturation of 74 % and tension of 40·8 mm Hg. This enables the partial pressure gradient for the next Δt sec to be found (131–40·8 mm Hg) and the process is then repeated.

After about 8 such steps, the blood tension has risen to the alveolar tension of 131 mm Hg. However, it can be shown that the blood remains in the capillary considerably longer than this. The mean alveolar–capillary gradient is given by oxygen consumption divided by an assumed diffusing capacity, i.e. 576/30 = 19 mm Hg. Some 28 intervals of Δt sec will be necessary for the mean gradient to reach this low value, so that the $^{16}O_2$ has virtually equilibrated about one third of the way along the capillary.

The calculation for $^{15}O_2$ is very similar. The initial partial pressure gradient is taken to be 131 mm Hg multiplied by a scaling factor C. The oxygen transferred in the first Δt sec is therefore

$$131C \times 0{\cdot}000107 = 0{\cdot}0140 \times C \text{ vol./vol.}$$

This is converted to the new tension by using

$$\frac{P_{15}}{C_{15}} = \frac{P_{16}}{C_{16}} = \frac{37{\cdot}5}{0{\cdot}138}$$

in the first Δt sec. The new gradient is therefore $(131-3{\cdot}8)C$, and so on.

The diffusion constants for $^{15}O_2$ and $^{16}O_2$ will not be identical, but the difference is very small and has not been measured. A formal difficulty in the above analysis is that the partial pressure of $^{15}O_2$ is a function not only of $^{15}O_2$ content but also of $^{16}O_2$ content, and, for each step of the integration, both these factors should be taken into account. However, it can be shown that in practice the error introduced by considering the effect of $^{15}O_2$ content only is very small.

(2) It is assumed that the end-capillary blood is equilibrated with respect to $^{15}O_2$ and $^{16}O_2$. Further, the amount of $^{15}O_2$ in the pulmonary capillary blood is constant (after the first second or so of breath-holding) so the rate at which $^{15}O_2$ leaves via the pulmonary vein is equal to the rate of leaving the alveoli. (This equality does not apply for $^{16}O_2$ because of $^{16}O_2$ in the mixed venous blood.)

At any instant t (during breath-holding following inspiration of the radioactive gas), the fractional rate of $^{15}O_2$ removed from the alveoli is equal to the rate at which *total* $^{16}O_2$ leaves via the pulmonary vein divided by the total alveolar $^{16}O_2$ at time t. If P_{15} and P_{16} are the partial pressures of $^{15}O_2$ and $^{16}O_2$ in the alveoli, k is the rate of O_2 consumption (assumed to be constant) and K is the reciprocal of barometric pressure less ambient water vapour pressure,

$$\frac{1}{P_{15}} \cdot \frac{dP_{15}}{dt} = \frac{\dot{Q}cC_{16}}{V_A P'_{16} K - kt},$$

where $k = \dot{Q}c(C_{16} - Cv_{16})$ and P'_{16} is the initial value of P_{16}.
After integration this gives

$$\frac{P_{15}}{P'_{15}} = \left[\frac{V_A P'_{16} K - kt}{V_A P'_{16} K}\right]^{\dot{Q}cC_{16}/k}$$

where P'_{15} is the initial value of P_{15}. This may be written $P_{15}/P'_{15} = (1-rt)^n$, where r and n are as explained in the text.

The corresponding expression for $^{16}O_2$ is

$$\frac{P_{16}}{P'_{16}} = 1 - rt.$$

Therefore the ratio R of $^{15}O_2$ to $^{16}O_2$ uptake is given by

$$R = \left(1 - \frac{P_{15}}{P'_{15}}\right) \div \left(1 - \frac{P_{16}}{P'_{16}}\right) = \frac{1-(1-rt)^n}{rt}.$$

It is readily shown that this expression becomes simply

$$R = n$$

for very short periods of breath-holding or for situations in which the rate of O_2 consumption, relative to the amount available, is small.

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CUTANEOUS MECHANORECEPTORS WITH AFFERENT C FIBRES

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The analysis of cutaneous afferent mechanisms by recording electrically from the afferent nerve fibres has been pursued vigorously since Adrian (1926) first developed suitable techniques. As a result there is now an extensive literature on, and general agreement concerning, the larger myelinated afferent fibres. However, the non-myelinated sensory fibres, because they are difficult to isolate as single units, have not been intensively studied, even though they are 4–5 times more numerous than the myelinated fibres in cutaneous nerves (Gasser, 1955). Adrian (1931) in the earliest work on the fine fibres had concluded that pain was carried by the smallest fibres because the stimuli required to excite a discharge of low-amplitude impulses from frog's skin were either strong pressure or chemicals which caused 'defensive movements' in spinal frogs. Zotterman in 1939, using improved techniques, recorded impulses of small amplitude from cutaneous nerves of the cat and he argued that they were carried by very small axons which were probably non-myelinated. Weak mechanical stimuli, such as lightly stroking the hairs, were sufficient to arouse a discharge of small impulses at low frequency, with a persistent after-discharge. Stronger mechanical stimuli and severe thermal stimuli caused a more abundant discharge of the small impulses in the multifibre strands which were used. Maruhashi, Mizuguchi & Tasaki (1952) were able, in toads, to prepare fine nerve strands containing only a few non-myelinated axons, and substantiated Zotterman's conclusion that some C fibres could be excited by lightly touching the skin. In addition, they found that there were other C fibres which were excited by severe thermal but not by mechanical stimuli. In the cat, although they could not keep the C fibres alive unless there were also A fibres in the strands, they recorded responses evoked by stroking the skin and when 'noxious and heat stimuli' were used. More recently, Douglas & Ritchie (1957*a*) recording from intact cutaneous nerves in the cat, have also confirmed Zotterman's observation by showing that C fibres were excited by stroking the skin. As many as 50 % of the C fibres examined by their technique could be excited by mechanical stimuli. They did not find any fibres excited by heating the skin, even when

their stimuli were sufficient to burn the fur. Many of the fibres excited by mechanical stimuli also showed a short-lasting response when the skin was cooled (Douglas, Ritchie & Straub, 1959).

Convincing comparisons of the activity in different sensory units cannot be made when the afferent impulses are recorded in multifibre preparations. Exact information about the properties of receptors can, however, be obtained by examining activity in single afferent fibres. Techniques for recording the activity in single afferent C fibres which were developed during work on the visceral afferent innervation (Iggo, 1957*a*, *b*; 1958*a*) have now been used, together with quantitative mechanical and thermal stimulation of the skin, to analyse the cutaneous afferent C fibres. This paper deals with the properties of the mechanically sensitive C sensory units; the thermoreceptive units are reported elsewhere (Iggo, 1959*b*; Hensel, Iggo & Witt, 1960). Preliminary accounts of the results have been published (Iggo, 1958*b*, 1959*a*).

METHODS

The cats used were anaesthetized with chloralose (80 mg/kg, I.V.) after induction of anaesthesia with ethyl chloride and ether. The right leg was abducted and fixed by two drill pins, one in each end of the tibia, so that the medial surface lay uppermost. The pins were inserted from the lateral side of the leg and care was taken to avoid any damage to the medial skin which is innervated by the saphenous nerve. The saphenous nerve was ligated in the groin, dissected free in the upper thigh, and covered with liquid paraffin B.P. which filled a trough formed by tying the edges of the skin wound to a metal ring. A black Perspex plate was placed under the nerve and the fine dissection (at $\times 40$) carried out with sharp needles and knives. Stimulating electrodes were placed under the saphenous nerve 20–30 mm distal to the recording electrodes. Further details of the recording and stimulating techniques and the methods used to identify single afferent fibres have been described (Iggo, 1958*a*). The thermal stimuli used were provided by metal rods (10 mm diam.) at the desired temperature, by small electrical radiators, and in the later experiments by thermodes with which the temperature could be altered as required without changing the cutaneous mechanical stimulus. The cutaneous temperatures were measured with thermistors (Stantel, Type F2311/300).

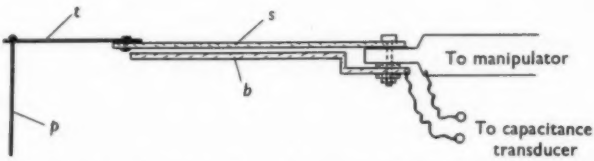

Fig. 1. Diagram to illustrate the mechanotransducer used to stimulate the cutaneous receptors. *s*, flat steel spring; *b*, flat brass plate of dimensions similar to *s*; *t*, tufnol rod screwed on to *s*; *n*, stiff nylon thread, 0·25 mm² tip area, attached to *t*. The unit was attached to a micromanipulator.

Mechanical stimuli. The mechano-transducer used is illustrated in Fig. 1. The nylon probe *p* (tip area 0·25 mm²) was attached to a rigid light rod of tufnol *t*, which was mounted on a flat steel spring *s*. The spring was fixed above, and insulated from, a flat brass plate of

similar dimensions, which in turn was fastened securely to a 10 mm diameter brass tube. The tube was attached to a micromanipulator which had calibrated movements in two directions at right angles to each other and parallel to the skin surface. In use, the probe was lowered on to the skin; as the downward movement continued after contact was made, the spring was deflected away from its resting position, so applying a force to the skin and altering the capacitance of the unit. The resistance of the spring to deformation and thus the sensitivity of the unit could be varied by using different springs. Calibration of the unit with an air-damped balance allowed the force exerted on the skin to be calculated. The voltage output of the condensor transducer (Alexander, 1951) was displayed on cathode-ray tubes for visual control and photography as required.

RESULTS

The receptors described in this paper were in the skin on the antero-medial surface of the lower thigh, the leg and the foot. The afferent fibres were dissected from the saphenous nerve in the upper thigh. There was a distance of at least 100 mm between the recording electrodes and the endings of the afferent fibre. Care was taken to avoid working on receptors in skin close to the wound in order to remove any risk of altered sensibility of the endings, such as the hyperalgesia reported by Lewis (1942). For the same reason, frequent exposure of the skin to noxious stimuli was also avoided. In some early experiments, the use of sharp pins for exploring the skin abolished the responses of single units.

The sensory units to be described were all excited by mechanical stimuli and were either not excited at all by thermal stimuli or gave a brief discharge of impulses when the skin was heated or cooled. They are classed as mechanoreceptors because of this differential sensitivity.

Conduction velocity of the afferent fibres

The conduction velocities of twenty-seven of the fifty-eight afferent fibres reported in this paper were measured by techniques already described (Iggo, 1958*a*). For nine of the fibres, two measurements of the velocity were made; one in the nerve trunk in the thigh and the second for conduction from the cutaneous endings of the fibre to the recording electrodes. For the latter measurement the stimulating electrodes were placed on the skin and the action potential identified by its similarities to action potentials evoked by natural stimuli. With the very fine nerve strands used, which contained only a few live fibres, this procedure was exact. Conduction velocities computed by the latter measurement were invariably slower than by direct measurement in the main trunk of the nerve. The average ratio for the nine fibres was 1·16:1. A similar phenomenon was reported for visceral afferent C fibres in the vagus (Iggo, 1958*a*) and in cutaneous myelinated fibres by Maruhashi *et al.* (1952). The remaining eight conduction velocities in Fig. 2*a* were measured from the skin to the recording electrodes. All the velocities were in the range

0·55–1·25 m/sec for mechanoreceptors; most of the velocities lay between 0·65 and 1·05 m/sec. If an adjustment is made for the slower conduction velocity in the distal part of the nerve these figures become 0·6 and 1·4 m/sec for conduction in the main saphenous nerve. These values are within the limits of the velocities of the slowest and fastest waves of the compound action potential of C fibres in the saphenous nerve reported by Gasser

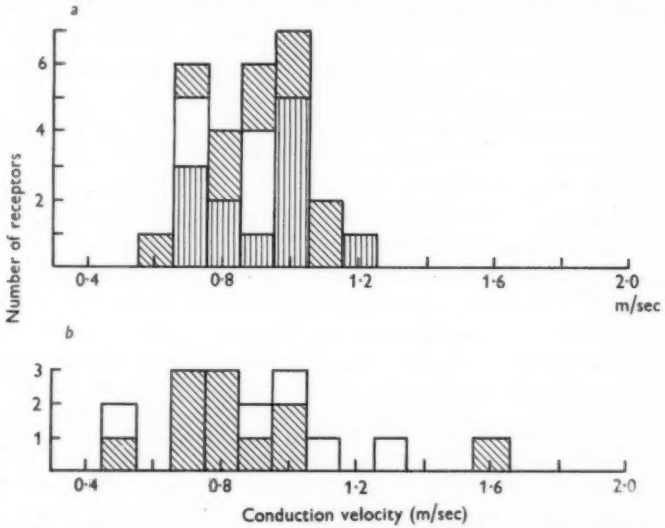

Fig. 2. The relation between the conduction velocity of single afferent fibres and the sensitivity of their endings in the skin. (*a*) Mechanically-sensitive afferent units; these are the units described in this paper. The units are divided into three groups, of low ▥, medium □ and high ▧ sensitivity, as described in the text. There is no obvious segregation according to mechanical sensitivity of the endings. (*b*) Temperature-sensitive units; ▧ heat-sensitive, □ cold-sensitive. These results are taken from Iggo (1959) and Hensel *et al.* (1960). The conduction velocities in this figure are for conduction from the endings in the skin to the recording electrodes.

(1950). One fibre with a conduction velocity of 2·6 m/sec was recorded in the present work but the receptor was not identified; this velocity is at the upper limit for conduction in C fibres according to Gasser's results.

There was no apparent relation between the mechanical sensitivity of the mechanoreceptors and the conduction velocity of the afferent fibres. Figure 2*b* shows that the same conclusion holds for the thermoreceptors; there is no preferred velocity for the thermoreceptors and, as with the mechanoreceptors, the majority lie between 0·65 and 1·05 m/sec. There does not seem to be any segregation according to the thermal sensitivity of the units either.

The conduction velocity of the remainder of the units in this paper were assessed either from less accurate measurements of the conduction time (six fibres) or from the general features of the response to stimulation of the receptors (twenty-six fibres). Three useful guides to the size of the fibres were: (*a*) the interval between stimulation of the receptor and the arrival of the first impulse at the recording electrodes, a few milliseconds for myelinated fibres and more than 100 msec for the C fibres; (*b*) the configuration of the impulse and in particular the sound produced when the amplified impulses were played through a loudspeaker; the C spikes were longer than the A spikes, and less sharply inflected; and (*c*) the presence of an after-discharge. The last was the least reliable guide, since although the afferent units with myelinated fibres conducting impulses as slowly as 9 m/sec did not exhibit an after-discharge, neither did all the C fibres. Assessments of conduction velocity based on these indirect measures are, of course, less reliable than the direct measurements, and the results from such units were used only to supplement the data obtained from twenty-seven units of known conduction velocity. Whenever a unit with a new type of response to natural stimulation was isolated, the conduction velocity of the afferent fibre was one of the first measurements made.

The response to mechanical stimulation of the skin

The standard stimulus used in the present work was pressure on the skin with a vertically applied nylon thread (cross-sectional area 0·25 mm^2). The load (a force of 5 g wt. in Fig. 3) reached a steady value within 100–500 msec of its application and the probe was kept at the final position until it was removed after some seconds (Fig. 3). The rate of application was not constant from trial to trial since the probe was lowered on the skin by manually turning the vertical movement of the micromanipulator on which the probe was mounted. The discharge of impulses in the afferent fibres reached its peak frequency within 100 msec of the first impulse and remained at or near the peak value for as long as the load was approaching the final value and for a short time after the steady value was reached. In the example shown (Fig. 3) there was an initial burst (peak frequency 60/sec) lasting 500 msec, after which the frequency of discharge fell quickly at first and then more slowly. In this figure the upper record has been moved to the left by a distance which equals the conduction time from the nerve endings to the recording electrodes. As a result, the relation between stimulus and response of the nerve-endings is shown more clearly. A similar response is shown in Fig. 5. The most persistent discharge of impulses was obtained when the probe was on the centre of the receptive field. At one trial the peak discharge was 50/sec, this fell to 16/sec after 1 sec, and 10 sec later it was 4/sec. With some units,

the discharge persisted at frequencies as high as 20/sec for 30 sec. This pattern of response was characteristic of the C fibres, an initial high-frequency burst of impulses during and immediately after application of the stimulus followed by a variable persistence of the discharge at a

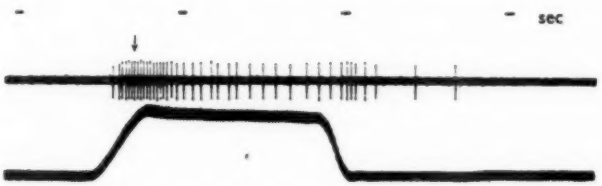

Fig. 3. The response of a C mechanoreceptor to mechanical stimulation of the skin. Initially the probe was stationary just above the skin and was then lowered and reached its final position in 350 msec. This position was maintained for 1 sec and the probe was then lifted clear of the skin. The initial burst of impulses reached a peak frequency of 60/sec as the load was being applied, after 1 sec the frequency had fallen to 12/sec. There was an off-burst as the probe was removed from the skin and a brief after-discharge.

In this figure the upper trace, which shows the impulses in a single C fibre, has been moved to the left by a distance which corresponds exactly with the conduction time from the nerve endings to the recording electrodes. The arrow indicates the *original* position of the first impulse. This has been done to show more exactly the temporal relation between stimulus and response. It has not been done for any other records in this paper.

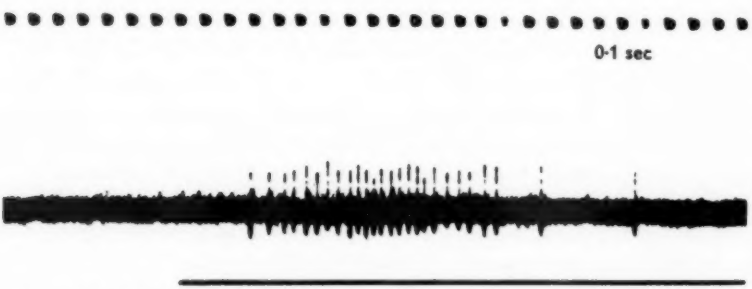

Fig. 4. The brief response of a low-sensitivity C mechanoreceptor to squeezing a fold of the skin with forceps. The stimulus indicated by the lower line was continued throughout the latter part of the record, at which time the unit was not responding.

gradually falling frequency during the steady maintenance of the stimulus. An example of a brief response is given in Fig. 4 which shows the discharge of impulses in the fibre from an insensitive mechanoreceptor in response to squeezing a fold of the skin with forceps.

Frequency of discharge in single C fibres

The peak frequency of discharge in response to natural stimulation was 100 impulses/sec. This high frequency, evoked only by high-intensity stimulation, was rarely maintained for more than a few impulses even with very rapidly applied loads. The peak frequencies in a sample of nineteen fibres ranged from 20 to 65 impulses/sec (mean 44). The sample included examples of all the categories enumerated in Fig. 7. There was no attempt to use maximal stimuli, nor were fibres firing at high frequencies selected. A more significant index of the activity in the C fibres is probably the total number of impulses carried in 1 sec. This ranged from 17 to 48 impulses (mean 24) for the same group of fibres, in the first second of stimulation. In some fibres the discharge persisted at 30 impulses/sec for several seconds during stimulation but more often the rate of firing had fallen to 5–10/sec, or had stopped, within a few seconds. Examples of single units are illustrated in Figs. 3, 4 and 5. Similar rates of firing were found for the thermoreceptors with afferent C fibres (Iggo, 1959*b*; Hensel *et al.* 1960) and for the visceral afferent C fibres (Iggo, 1957*a*, *b*).

Repetition of brief mechanical stimulation sets up an irregular discharge of impulses. For example, stroking the skin 4 times/sec gave frequencies in a single fibre fluctuating between 5 and 50 impulses/sec.

The rate of discharge in small myelinated fibres has also been recorded. With small A fibres (e.g. an A fibre with a conduction velocity of 9 m/sec) the frequency exceeded 200 impulses in the first second of stimulation. As with the C fibres there was an intermittent discharge when the skin was stroked repetitively; the bursts of impulses were, however, even more distinct than with the C fibres.

Size of the receptive field

The receptive field in these experiments is defined as that area of skin from which a steady discharge of impulses could be evoked in the afferent fibre by the weakest stimulus to the skin. The fields as measured probably over-estimate the area of skin in which the sensitive nerve endings were present, because the spread of the stimulus through the skin was unavoidable.

The field was measured in ten fibres by mapping the response of the individual unit to punctate stimulation with the 0·25 mm^2 probe, mounted on a micromanipulator. The pressure was monitored in order to produce a standard stimulus. Typical results are illustrated in Fig. 5. At the most sensitive spot on the skin the discharge appeared with the shortest latency, the highest frequency and the slowest adaptation. When the probe was moved away from such a spot there was a smaller response

until with the probe 3 mm away from the centre of the field, the unit failed to fire unless the stimulus intensity was increased. The influence of the position of the stimulus on the latency of the response is shown in Fig. 5*b*. At the centre of the field the latency was 145–165 msec, and if the conduction time (130 msec) is subtracted, this is reduced to 15–35 msec. The latency 2 mm away had increased to more than 400 msec.

A flat surface was necessary for accurate mapping and the most successful experiments were those in which the receptor was in the medial skin of the thigh or leg. For the ten carefully mapped units the fields measured from 2×2 to not more than 5×5 mm^2. In seven other fibres the fields were mapped less accurately, but similar dimensions were found. No unit was found with a larger receptive field. However, for the hair receptors and the most sensitive mechanoreceptors, the apparent field was sometimes as large as 50×50 mm^2. The size depended on the intensity of stimulation. Such apparently large fields resulted from the use of unmonitored stimuli. Slight movements caused by exploring the skin and hair were sufficient, with the most sensitive units, to excite endings in skin several centimetres away from the probe. Progressive reduction of the stimulus led eventually to the accurate location of the usual small field.

There was never more than one small part of the skin surface from which a discharge of impulses could be evoked by a stimulus about twice threshold. The single C fibres presumably, therefore, terminate in a small area of skin and any branching must occur close to the terminations.

Rate of adaptation

The discharge of impulses provoked by pressing steadily on the centre of the receptive field of a C mechanoreceptor did not persist indefinitely even when the stimulus was well above threshold. The following result is typical of many of the more sensitive units. There was an initial burst of impulses at 60–70/sec as the load was being applied. After 1 sec the frequency had fallen to 25/sec. This quick phase of adaptation was succeeded by a more gradual fall in the frequency. At 1·5 sec, the rate was 16/sec and after 10 sec it was 4/sec. Other units adapted more slowly: 20 sec after the load was applied one unit was firing at 30/sec. Still other units adapted more quickly, e.g. Fig. 4 shows a response to squeezing the skin which lasted only 1·5 sec even though the stimulus was maintained.

The A hair receptors and the A touch receptors adapted much more quickly than the comparable C fibres. The slowly-adapting A pressure receptors in the cat and rabbit, on the other hand, have a very much more persistent discharge and were still firing after 5 min of steady stimulation (Frankenhaeuser, 1949; N. Fjällbrant & A. Iggo, unpublished experiments).

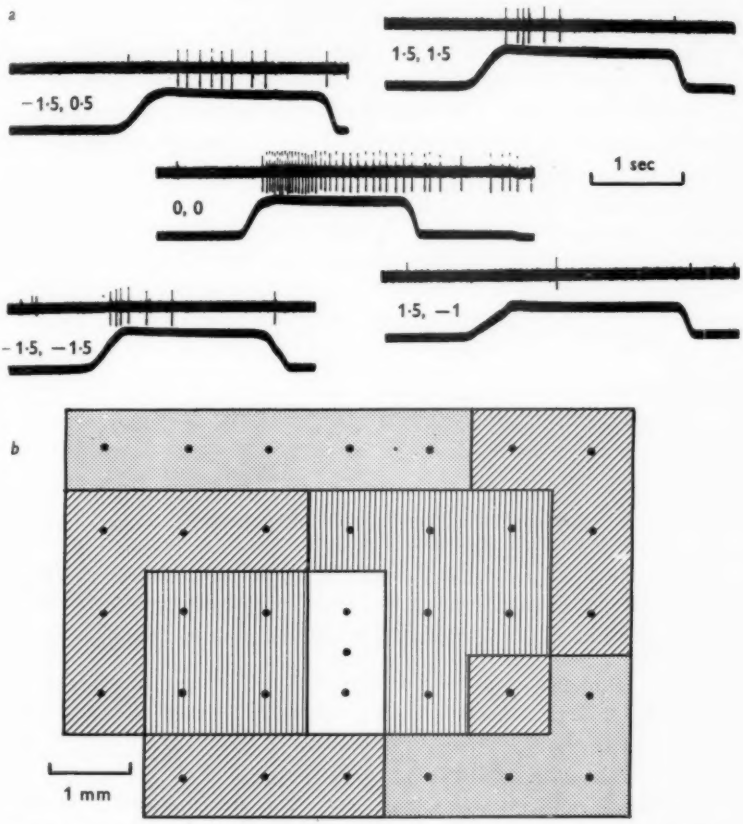

Fig. 5. The receptive field of a C mechanoreceptor. (*a*) shows five records obtained when different spots on the skin were touched with the probe. The final load is nearly the same (2 g wt.) for all positions. The distances of the probe from the most sensitive central position are indicated in millimetres. (*b*) is a diagram summarizing all the results for the same unit. The skin was stimulated on a 1 mm grid and each spot is indicated by a filled circle in the diagram. The latency of the first impulse at each position is shown by the shading. At the centre of the field the latency was least, the rate of firing was highest and the persistence of the discharge was longest. The following meaning is given to the shading:

	Latency (msec)	Impulses in first 1·5 sec
□	145–165	35
▥	200–350	22
▨	440–750	6
▤	—	0

After-discharge

A characteristic feature of the C mechanoreceptors, especially the more sensitive examples, was the persistence of the discharge of impulses for as long as 10 sec following a brief stimulation of the skin. It was especially prominent when the skin was stroked (Fig. 6). In this figure, the peak frequency of discharge was similar when the skin was stroked and when it was pressed. The after-discharge, however, was present only after stroking. Another example is shown in Fig. 8*b*; there was a persistent after-discharge in C fibres when the tips of the cat's fur were stroked. The rate of firing

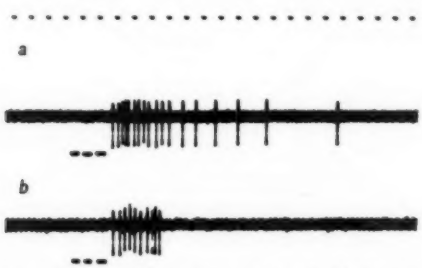

Fig. 6. The response of a C mechanoreceptor (threshold, 50 mg wt.) to (*a*) stroking the skin and (*b*) pressing briefly on the skin with the nylon probe. The dashes indicate the approximate duration of the stimuli.

during the after-discharge was always low. The peak frequency could reach 100/sec when the load was being applied. After removal of the load the frequency of the after-discharge was about 5/sec initially and as it was disappearing the rate would fall to less than 0·5/sec. An especially pronounced after-discharge was found when the skin had been left undisturbed for as long as 30 min. At the same time there was an enhanced excitability of the endings to mechanical stimulation.

Inexcitability as a consequence of repeated stimulation

When a mechanical stimulus was repeated frequently at short intervals, the response to successive stimuli was progressively reduced. This effect was more evident when the stimulus was the nylon probe than when the skin was stroked. In a typical example the receptor was stimulated by pressing on the skin 10 times in 13 sec. The number of impulses evoked by the successive stimuli were 7, 2, 3, 3, 1, 2, 1, 0, 0, 0. One minute later the unit could be excited again by a similar stimulus. Similar stimulation of another unit for 25 sec abolished the response to mechanical stimulation for 4 min, after which there was a gradual recovery of the initial sensitivity. At 15 min the after-discharge reappeared and the response was almost

restored to its original value. Stroking the skin for 30 sec did not alter the immediate response of this same unit to stroking the skin, but did lessen the after-discharge. Rubbing the skin abolished the response to mechanical stimulation.

When an interval of at least 30 sec was left between successive stimuli, the response to a standard stimulus was uniform. More frequent stimulation led to a progressive fall in the response.

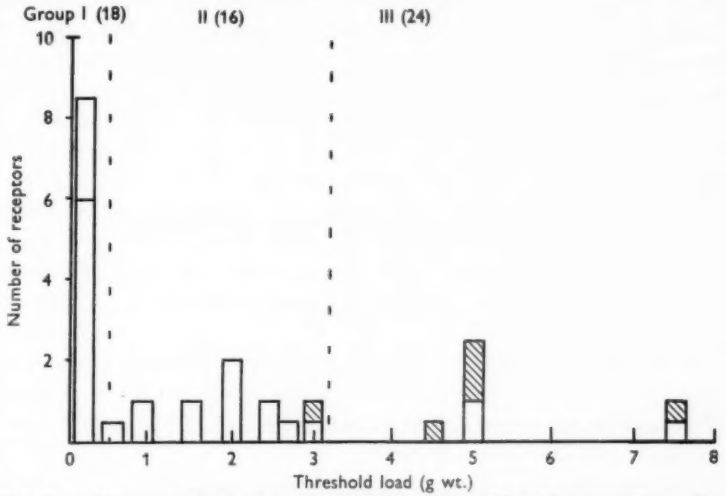

Fig. 7. A histogram showing the threshold values for 24 mechanoreceptors. The hatched areas ▨ refer to 6 thermoreceptors (data from Iggo, 1959*b*). The interrupted vertical lines at 0·5 and 3·1 g wt. indicate the boundaries chosen in the classification of the mechanoreceptors into three groups, I, II, and III according to sensitivity (see text). The numerals in parentheses give the total number of units of the appropriate sensitivity which were found. They include, in addition to the units the sensitivity of which was measured, receptors whose sensitivity was assessed from less accurate tests.

Sensitivity of the mechanoreceptors

There was a wide range in the sensitivity of the mechanoreceptors to mechanical stimulation, as is indicated by the thresholds which ranged from 25 mg wt. to 5 g wt., a ratio of more than 200:1. Figure 7 is a histogram showing the frequency distribution of all the units for which there were threshold measurements. It probably does not represent a fair sample of the mechanoreceptors because the most sensitive endings were the easiest to find. An alternative way to describe the sensitivity of the receptors is in terms of their response to the mechanical stimuli to which the skin is normally exposed and which are also commonly employed in the experimental analysis of reflexes initiated from the skin. These are

touching, stroking, rubbing, pinching, etc. The following division into three categories, although arbitrary, is useful in indicating the wide range in sensitivity of the C mechanoreceptors.

1. *Highly sensitive endings* (18 *fibres*). This group includes the receptors to the left in the histogram in Fig. 7 and a boundary at 500 mg wt. has been chosen. The most sensitive of these units had a threshold of 25 mg wt. Some of these very sensitive units could be excited by touching the tips of the hairs, whereas other equally, or even more, sensitive to direct pressure on the skin could not be excited by similar movements of the hairs. The C hair receptors were excited by moving only the tips of hairs, and the rate of discharge in response to such mild stimulation was surprisingly high; e.g. for one unit the discharge was at 25/sec when the tips of the hairs were brushed and was not more than doubled by a firm stroke of the skin. Another unit gave a burst of impulses lasting 1·5 sec, with a peak frequency of 32/sec, when the hairs were bent backwards. This persistent discharge from the C hair receptors when they were moved from rest to a new position and held there is quite characteristic and very different from the brief burst of impulses at high frequency from the A hair receptors (Fig. 8).

2. *Medium sensitivity* (20 *fibres*). Units excited by stimuli between 0·5 and 3 g wt. are placed in this group. These units were not excited by lightly touching the fur or lightly stroking the skin. A firmer stroke of the skin or light pressure was required.

3. *Low sensitivity* (24 *fibres*), threshold greater than 3 g wt. These units were not excited easily by a firm stroke along the skin with a smooth rod. Firm pressure with the points of forceps or a sharp pin or squeezing a fold of the skin, or pulling the hairs was required. The usual response was a burst of impulses lasting 1–2 sec, e.g. Fig. 4.

Comparison with A mechanoreceptors

The cutaneous receptors innervated by myelinated (A) and by non-myelinated (C) fibres have overlapping thresholds for mechanical stimulation. The thresholds of the most sensitive A touch and A hair receptors were too low to be measured with the transducer used to excite the C mechanoreceptors; they are therefore more sensitive than the most sensitive C mechanoreceptor yet found. Many A pressure receptors had thresholds of more than 1 g wt., i.e. higher than the most sensitive mechanoreceptors, and Frankenhaeuser (1949) has reported similar thresholds for slowly-adapting pressure receptors with afferent fibres in the rabbit's sural nerve. There are still other A pressure receptors which were not excited by loads of less than 5 g wt. (A. Iggo & N. Fjällbrant unpublished observations). Some of these differences are illustrated in

Fig. 8, which shows the activity in a multifibre strand of the saphenous nerve. Very weak stimuli excited only A hair fibres, stronger stimuli brought in C hair fibres and pulling the hairs added one A fibre (with the largest spike in the tracing, an A pressure receptor) together with several other unidentified units. The A pressure receptor could also be excited by pressing on the skin.

The effect of thermal stimuli

If the mechanoreceptors were excited at all by heating the skin, they required much higher temperatures of the stimulating rod than did the

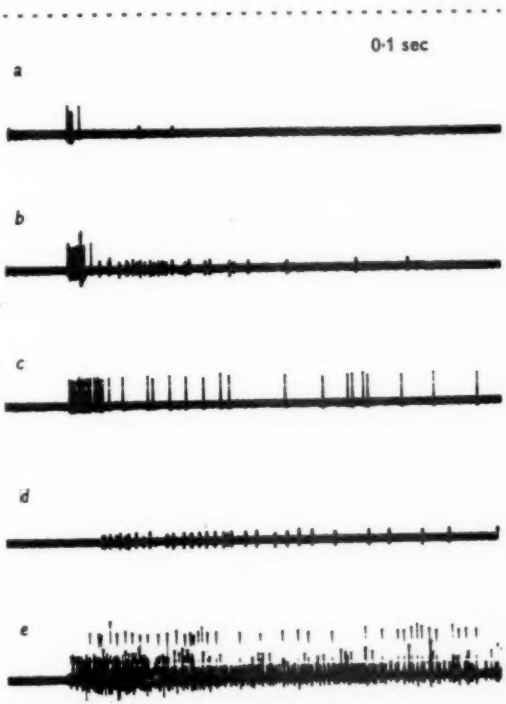

Fig. 8. Impulses in a multifibre strand of the saphenous nerve. *a* and *b* show the response to stroking the tips of the hairs, lightly in *a* and more firmly in *b*. In *a* there is a brief high-frequency burst of impulses in a myelinated fibre; this burst is longer in *b* and in addition there is a later more prolonged burst of impulses in at least two C fibres. These records illustrate the greater sensitivity of the A hair receptors. *c* and *d* show the response to bending the hairs and holding them in their new positions; *c* for the A hair fibre and *d* for the C hair fibres. The discharge in the non-myelinated fibres persists at a relatively higher frequency than in the A fibre. *e* illustrates the effect of pulling on the hairs. Several additional units are now firing, including an A pressure receptor (largest spike).

heat receptors (Iggo, 1959*b*). There was a brief discharge of impulses lasting perhaps 1 sec when rods at 80–100° C touched the skin. The units thereafter failed to respond to previously effective mechanical stimuli and also failed to be excited by the heat. Presumably the nerve endings had been damaged by the high temperatures needed to provoke a response. The heat receptors on the other hand gave consistent responses with much milder thermal stimuli, and continued to respond to appropriate stimulation for at least 3 hr (Iggo, 1959*b*). Zotterman (1936) has also recorded this effect of high temperature in abolishing the response of A mechanoreceptors to mechanical stimulation at the same time as it provoked a discharge of small impulses in other fibres.

TABLE 1. The relative proportions (%) of different types of cutaneous receptors innervated by C fibres

	Total sample of C fibres (97 fibres)						
Fibres ...	Mechanoreceptors 58			Thermoreceptors 39			
				Heating skin		Cooling skin	
Sensitivity ...	High	Medium	Low	High	Low	High	Low
	31	28	41	8	60	16	16

(Data from Iggo, 1959*b*; Hensel *et al.* 1960; and the present paper.)

Cooling the skin may excite C fibres which can also be stimulated by mild mechanical stimulation (Douglas *et al.* 1959). When single C mechanoreceptors were examined, the discharge of impulses excited by cooling the skin was brief and at a low frequency. In order to demonstrate the effect of cooling, it was necessary to ensure that the thermal stimulus was not contaminated by any mechanical component, otherwise the much more effective mechanical stimulus masked the response. Thermodes were used in the most successful experiments. The rate of firing in response to sudden cooling of more than 4° C was less than 5/sec, compared with more than 50/sec in response to mechanical stimulation (see also Hensel *et al.* 1960). In contrast, the C cold fibres responded with a regular discharge of impulses which continued at least 30 min (Hensel *et al.* 1960).

The thermoreceptors had very high thresholds for mechanical stimulation; they fell into category III of the mechanoreceptors, but could be distinguished from the latter by their responses to thermal stimuli.

DISCUSSION

The single fibre results reported in this paper permit unambiguous statements to be made about some of the cutaneous receptors innervated by non-myelinated fibres. The receptors are not uniformly sensitive to all

cutaneous stimuli nor are they excited only by noxious or injurious stimuli. The results are in agreement with those of Zotterman (1939), Maruhashi *et al.* (1952) and Douglas & Ritchie (1957*a*), in showing that some of the C receptors have a high mechanical sensitivity. They go further, however, in establishing a wide range in the sensitivity of the individual units.

Douglas *et al.* (1959) have re-opened the question of the specificity of the afferent nerve endings by their suggestion that the excitation of the same C fibres by touch and by cooling is an example of dual specificity. Their methods, however, do not allow the responses of individual units to be followed. When single C fibres were examined the mechanoreceptors and the thermoreceptors exhibited significant differences in their responses to the same stimuli, particularly with respect to thresholds, rates of firing and the persistence of discharge during constant stimulation.

Table 1 gives a summary of all the results with single afferent C fibres which have been obtained in the recent series of investigations. The sample is biased, first, because among the mechanoreceptors the most sensitive were the easiest to find, and secondly, in the work on thermoreceptors reported by Hensel *et al.* (1960) the thermoreceptors were selected and the mechanoreceptors were discarded. It is evident, however, that the mechanoreceptors, as Douglas & Ritchie (1957*a*) have suggested, are the predominant type. There are also numerous thermoreceptors with C afferent fibres.

There was no apparent relation between the conduction velocity of the afferent fibres and either the degree or kind of sensitivity of the nerve endings to natural stimuli (Fig. 2). The scarcity of fibres of the slowest conduction velocity in the sample may reflect either the greater difficulty of isolating them as single units, or a real difference in the specificity of the endings. Douglas & Ritchie (1957*a*) also had difficulty in exciting the slowest fibres with mechanical stimuli.

The rate of firing in individual C fibres is shown by the present work to be lower than in the myelinated cutaneous afferent fibres, where it may exceed 800 impulses/sec in response to natural stimulation of the receptors. Direct recording of the impulses in single C fibres during natural stimulation of both visceral (Iggo, 1957*a*, *b*) and cutaneous (Iggo, 1959*b*; Hensel *et al.* 1960; this paper) afferent fibres has revealed rates of firing up to 100 impulses/sec. Douglas & Ritchie (1957*b*) using an indirect method estimated that the 'maximum rate at which mammalian non-medullated cutaneous afferent fibres fired in response to touch was low'—not faster than 10 impulses/sec. This very low estimate may have been due to the population of C fibres failing to respond in the uniform manner assumed in their analysis. The stimuli which they used would be likely to produce

widely fluctuating rates of discharge in an inconstant sample of the population which they were testing.

Previous estimates of the size of the receptive fields for C fibres have relied on extrapolation from work on myelinated afferent fibres (Tower, 1940; Bishop, 1946), and led to the expectation that they would be large. The present results show that individual C fibres have quite limited fields. The mechanisms of cutaneous localization may therefore be simpler than has hitherto been assumed, since there is probably much less overlap of receptive fields than was expected.

The non-myelinated cutaneous fibres can no longer be regarded as exclusively nociceptive in function, since the most sensitive C mechanoreceptors and C thermoreceptors are comparable in sensitivity with touch and temperature sense in man. There is, however, a wide range in the thresholds for natural stimulation within the group of non-myelinated cutaneous afferent fibres. An attempt to identify the nociceptors by making close-arterial injections of allegedly pain-producing chemicals into the skin was inconclusive (Fjällbrant & Iggo, 1959).

SUMMARY

1. Electrical activity was recorded in single afferent fibres, with conduction velocities ranging from 0·55 to 1·25 m/sec, dissected from the saphenous nerves of cats anaesthetized with chloralose.

2. The cutaneous receptors were excited by mechanical stimuli, and the threshold loads ranged from 25 mg wt. to 7·5 g wt. The receptors were insensitive to heat and gave a brief discharge when the skin was cooled.

3. The discharge in individual fibres reached peak frequencies of 100/sec and persisted at rates as high as 50/sec for 1 sec. This discharge had an initial quick phase of adaptation, lasting less than 1 sec followed by a slow phase which, exceptionally, lasted for 30 sec. Frequent repetition of stimulation led to a temporary inexcitability of the ending.

4. The receptive fields of the receptors were small, ranging from 2×2 mm^2, to not more than 5×5 mm^2.

I wish to thank Mrs N. Fjällbrant for her help with some of the experiments and Miss W. Sandie for her willing assistance throughout.

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A COMPARISON OF METHODS FOR MEASURING EFFLUX OF LABELLED POTASSIUM FROM CONTRACTING RABBIT ATRIA

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Methods for measuring the efflux of radioactive tracers from tissues are based on one of two principles. The first is that of measuring the activity of tracer-loaded tissue, while immersing it in flowing inactive saline (Keynes & Lewis, 1951; Creese, 1954; Rayner & Weatherall, 1957, 1959; Persoff, 1958, 1959). The second involves counting the activity of samples of the initially inactive saline in which the labelled tissue is immersed (Hodgkin & Keynes, 1955; Rayner & Weatherall, 1957; Creese, Hashish & Scholes, 1958; Klein & Holland, 1958; Persoff, 1959; Keynes & Swan, 1959).

Studies on rat diaphragm by both methods (Creese, 1954; Creese *et al.* 1958) agree in giving efflux curves which do not deviate grossly from simple exponentials, and whose slopes appear to be similar. However, efflux of ^{42}K from the spontaneously contracting right rabbit atrium shows marked deviation from simple exponential kinetics (Rayner & Weatherall, 1957; Persoff, 1958), even under steady state conditions (Rayner & Weatherall, 1959). This paper reports results of experiments in which direct and effluent methods were used simultaneously to measure ^{42}K efflux from individual atria.

METHODS

The methods used were those of Rayner & Weatherall (1957, 1959) and Persoff (1958), with the following modifications: the salt solution used contained (mM): Na 145, K 5·3, Ca 1·7, Mg 1·2, Cl 128, HCO_3 25, H_2PO_4 1·2, dextrose 11. The solution was gassed before and during use with 95% O_2 + 5% CO_2. The pH was 7·4, as checked with a Pye Universal pH meter, model 1066.

A rabbit was killed by a blow on the head and bled. After removing the heart, the right atrium was dissected free, partly in cold saline and partly in air. The atrium was then stretched slightly and mounted isometrically as a horizontal triangular sheet on a rigid frame incorporating a piezo-electric device for measuring the rate of spontaneous contraction. The procedure took 5–10 min. Particulars of the frame, the direct-counting bath and the counting apparatus have been given by Rayner & Weatherall (1957, 1959). Temperature was maintained at 37° C by means of a pump perfusion system. With saline flow rates between 5 and 180 ml./min, temperature in the apparatus varied less than ±0·5° C.

To keep saline flow above the critical rate for the 'open' system (experimentally determined to be 20 ml./min), and for convenience in computing effluent activity, it was desirable to maintain a constant saline flow rate. This was achieved by using an all-glass constant

pressure device (Fig. 1) designed on lines suggested by Vaughan Williams (1955). Gas was blown into the reservoir through the tube *A* where it was distributed through the saline, spread being facilitated by a sintered glass tip to the tube. After leaving the reservoir via *B*, the gas was conveyed through polythene and glass tubing to a point well below the surface of a fixed volume (about 50 ml.) of saline in a 100 ml. measuring cylinder. The cylinder was mounted at a higher level than the reservoir. From here the gas escaped into the atmosphere. Constant intra-reservoir pressure, and therefore uniform saline flow rate, depended on the existence of a fixed volume of liquid in the cylinder and the maintenance of a flow of gas through the apparatus. The saline outlet of the reservoir was connected, via a 100 ml. glass coil immersed in a water-bath at 37° C, to the saline inlet of the counting bath with polythene tubing. With the constant-pressure device, saline flow rate through the counting bath (maintained at 30 ml./min) was independent of the volume of saline in the

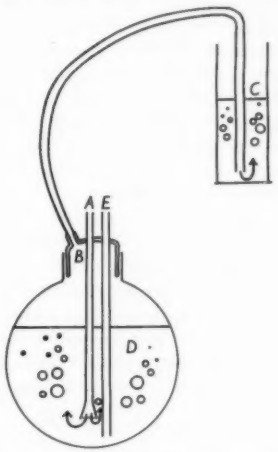

Fig. 1. All-glass apparatus for producing a constant rate of saline flow. *A* = gas inlet; *B* = gas outlet; *C* = measuring cylinder mounted above saline reservoir, *D*, and containing a fixed volume of liquid; *E* = saline outlet to atrium counting bath. Provided that the liquid level in the cylinder does not change, and gas is flowing through the apparatus, then saline flow rate is constant.

reservoir. The total alteration in saline flow rate as the reservoir emptied and when the collecting beaker was changed was less than ±1 % (S.E. of mean of 100 measurements).

Atria were equilibrated in inactive saline for 120 min before being placed in saline containing ^{42}K prepared from irradiated spectroscopically pure K_2CO_3 (A.E.R.E. Harwell), but otherwise identical in composition to the inactive saline. Exposure to ^{42}K was usually carried out in a side bath. The percentage exchange of tissue potassium at the end of the uptake period was estimated by the method used by Rayner & Weatherall (1957). In this type of experiment the lower part of the atrium frame was dipped rapidly in a beaker of gassed inactive saline at 37° C before beginning the efflux measurements, to avoid contaminating the effluent with active saline that had remained on the frame. In some experiments measurements by direct counting were made throughout the uptake period, by the method described by Persoff (1958). Efflux was measured by counting tissue activity directly by the method of Rayner & Weatherall (1959). In any one period of counting at least 5000 counts were registered. In the same experiments in which tissue activity was counted directly, effluent samples were collected at 5 min intervals in 200 ml. beakers situated immediately

below the saline outlet. There was thus virtually no 'dead space' between the collecting beaker and the site of tracer exchange.

When measurement of efflux was finished, the atrium was rinsed rapidly with isotonic choline chloride, blotted, and weighed before and after drying in a 15 ml. 'Pyrex' centrifuge tube in an air oven at 110° C during the night. The ashing technique used, with nitric and perchloric acids (1 ml. of 10:1 (v/v); British Drug Houses' 'Analar' reagents), was modified from that described by Rayner & Weatherall (1959). A controlled temperature of not more than 180° C was found to be necessary, or else potassium was lost to, and sodium gained from, the glass tube.

Potassium in solutions of ashed atria and diluted saline was estimated by using an 'EEL' flame photometer (Evans Electroselenium). Standards were prepared containing appropriate concentrations of sodium. Recovery of added potassium was $99{\cdot}4 \pm 2{\cdot}8\,\%$ (S.E. of mean for 8 determinations). Activities of solutions of atria, diluted saline and effluent samples were counted in a liquid counter (cf. Rayner & Weatherall, 1957, 1959). Usually at least 5000 counts were registered for each sample. The activities of the later effluent samples, however, were too low for this to be practical and only 1000 counts were registered. All counts (direct and effluent) were corrected for apparatus 'dead time', background (< 12 counts/min) and isotope decay. Tracer was used within 72 hr of being removed from the atomic pile. After calibration of the direct-counting apparatus against the liquid counter, the amount of activity lost by a given atrium was found to agree within $\pm 3\,\%$ (S.E. of mean of 15 results) with the amount of activity gained by the effluent.

Efflux regressions and rate constants were computed by the method of 'least squares', with 5 consecutive values of $\log_e$ (counts/min). No correction was applied for the difference in precision of early and late counts.

Several right atria were stretched on frames and fixed in formalin immediately after dissection. They were embedded in resin, sectioned (8 μ thick) and stained as described by Miles & Linder (1952). Diameters of cells were measured to the nearest 0·5 μ with a microscope with a calibrated $\times 20$ eyepiece and $\frac{1}{16}$ inch (1·5 mm) oil-immersion objective. In addition, sections of right atria prepared by Rayner & Weatherall (1957) were re-examined.

THEORY

The outward movement of ^{42}K can be measured by two methods ('direct' and 'effluent'). The relation between the two is required for the case where the semilog. activity–time curves are not linear, but the sum of two exponentials. In such a system,

$$f = a e^{-k_1 t} + b e^{-k_2 t}, \tag{1}$$

where f = total tissue activity (dimensions = counts/min) remaining after time t, and represents values obtained by the direct method; k_1 and k_2 are rate constants for ^{42}K exchange (dimensions = min^{-1}); a and b are constants with the same dimensions as f.

The activity of the effluent in a given time is equal to that lost by the atrium in the same time. Its values are thus minus the differential of equation (1). If E = rate of loss of tissue activity (dimensions = counts/min^2), then

$$E = -\frac{df}{dt} = a k_1 e^{-k_1 t} + b k_2 e^{-k_2 t} \tag{2}$$

(cf. Keynes & Swan, 1959).

The values of f in equation (1) are plotted on semilogarithmic paper to obtain slopes from which k_2 and (by subtraction) k_1 are obtained (cf. Rayner & Weatherall, 1959). The slope of the semilog. activity–time curve for the direct counts is the differential of the logarithmic of equation (1), or

$$S_1 = \frac{\mathrm{d}}{\mathrm{d}t}(\log f) = -\left[\frac{ak_1\mathrm{e}^{-k_1t} + bk_2\mathrm{e}^{-k_2t}}{a\mathrm{e}^{-k_1t} + b\mathrm{e}^{-k_2t}}\right]. \tag{3}$$

The slope of the semilog. activity–time curve for the effluent fluid is

$$S_2 = \frac{\mathrm{d}}{\mathrm{d}t}(\log E) = -\left[\frac{ak_1^2\mathrm{e}^{-k_1t} + bk_2^2\mathrm{e}^{-k_2t}}{ak_1\mathrm{e}^{-k_1t} + bk_2\mathrm{e}^{-k_2t}}\right], \tag{4}$$

and

$$|S_2| - |S_1| = \left[\frac{ab(k_1 - k_2)^2\ \mathrm{e}^{-(k_1+k_2)t}}{[ak_1\mathrm{e}^{-k_1t} + bk_2\mathrm{e}^{-k_2t}]\,[a\mathrm{e}^{-k_1t} + b\mathrm{e}^{-k_2t}]}\right]. \tag{5}$$

Since the quantity inside the brackets must be positive for all values of k_1 and k_2 (or zero when the k's are equal), the slope S_2 must be greater than S_1.

Corollaries

1. *At $t = 0$*

From equations (3) and (4),

$$S_1 = -\left[\frac{ak_1 + bk_2}{a + b}\right], \tag{6}$$

and

$$S_2 = -\left[\frac{ak_1^2 + bk_2^2}{ak_1 + bk_2}\right]. \tag{7}$$

Now if $ak_1 \gg bk_2^2$, then

$$S_2 \simeq -k_1. \tag{8}$$

2. *At $t \to \infty$*

Referring to equation (3) and multiplying top and bottom by e^{k_2t}, then

$$S_1 = -\left[\frac{ak_1\mathrm{e}^{(k_2-k_1)t} + bk_2}{a\mathrm{e}^{(k_2-k_1)t} + b}\right] \tag{9}$$

for *all* values of t. As t tends to infinity, S_1 tends to $-k_2$. Treating equation (4) similarly it can be seen that S_2 also tends to $-k_2$.

3. *Value of a and b*

If the values of tissue activity at successive times of determination of efflux (corresponding to values of f in equation (1)) are plotted semilogarithmically against time, then the intercept on the f axis of the linear late part of the curve shows the value of a/b. When the effluent values (corresponding to values of E in equation (2)) are similarly plotted, then the intercept on the E axis of the linear late part of the curve shows the value of ak_1/bk_2.

Theoretical curves have been constructed by plotting $f/f_{t=0}$ and $E/E_{t=0}$

semilogarithmically against t, for $k_1 = 0{\cdot}0375$, $k_2 = 0{\cdot}0093$, $a = 0{\cdot}78$ and $b = 0{\cdot}22$. These are illustrated in Fig. 3 and demonstrate the points made in this section.

A double exponential can result from a parallel arrangement of two compartments of tissue potassium (Gellhorn, Merrell & Rankin, 1944). Provided that potassium exchange was complete at the beginning of efflux, then a and b are the sizes of the potassium fractions exchanging with rate constants k_1 and k_2, respectively. If tissue potassium was not fully exchanged at the beginning of efflux, then the above mathematics still apply, but the relative magnitude of a will be over-estimated if $k_1 > k_2$, or under-estimated if $k_2 > k_1$.

A double exponential can equally well result from a series arrangement of two compartments of tissue potassium (Cohn & Brues, 1945). In this case a and k_1, b and k_2 are related in a more complex way than in the parallel arrangement to the relative sizes of, and exchange rates in, the two compartments.

RESULTS

Potassium contents of atria

After isolation, right atria lose 5–10% of their intracellular potassium, reaching a new steady state in 100–120 min (Table 1). Before measuring tracer movements in the experiments to be described, atria were equilibrated

TABLE 1. Effect of time of immersion in saline on potassium contents of right atria

Time (min)	0	120	240	360	480
No. of atria	9	9	9	9	9
Mean K_i (μmole/g dry wt.)	350	336	318	324	320
S.E. of mean	±4	±5	±9	±14	±12

The loss of potassium at 120 min is significant ($P < 0{\cdot}05$); thereafter there is no further significant loss.

in inactive saline for 120 min. The assumption is made that individual atria were in a steady state of potassium exchange after this time. Following the period of equilibration, no deterioration of spontaneous activity was observed during experiments. The rate of contraction of different right atria varied between 170 and 190 per minute.

Dimensions of atrial cells

The frequency distribution of the diameters of 536 cells from 4 right atria is unimodal (Fig. 2), with a geometric mean size of $6{\cdot}6\ \mu \pm 0{\cdot}19\ \log_e$ units (calculated as described by Creese, Neil & Stephenson, 1956). Two observers measured the diameters of approximately the same number of cells. There was no statistical difference between the two sets of data ($P > 0{\cdot}5$) and the results were combined. Cell dimensions (fibre length and diameter) did not change with time of immersion in saline ($P > 0{\cdot}3$).

Uptake of ^{42}K

The two methods used to measure the uptake of ^{42}K—a single measurement on a given atrium (Rayner & Weatherall, 1957) and successive measurements on the same atrium (Persoff, 1958)—gave results which did not differ significantly, as is shown in Table 2.

A curve of the uptake of ^{42}K with the successive counting method, plotted according to the model of Harris & Prankerd (1957), is illustrated in Fig. 5. Its significance in this form is considered in the general discussion.

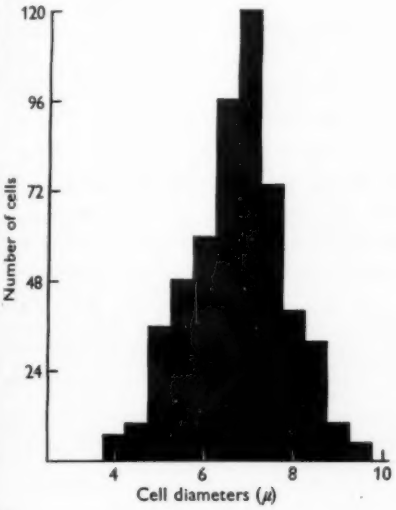

Fig. 2

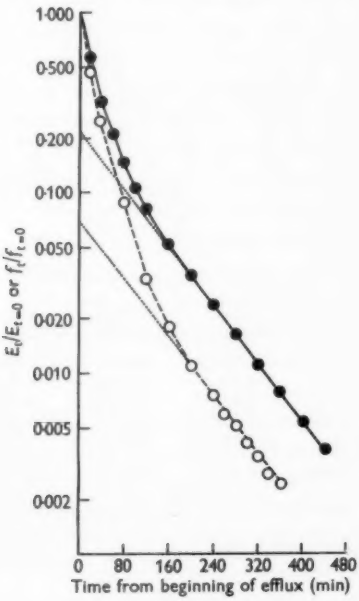

Fig. 3

Fig. 2. Distribution of diameters of 536 cardiac muscle cells from four right atria. Rabbits were killed and atria placed in fixative immediately after dissection and stretching on frames of dimensions similar to that used in efflux experiments. Note the unimodal distribution; geometric mean size 6·6 μ.

Fig. 3. Comparison of experimental ^{42}K efflux curves and theoretical curves for the double exponential. ●, tissue activity of Expt. 3; ○, effluent activity from the same expt. The constants $k_1 = 0{\cdot}0375$, $k_2 = 0{\cdot}0093$, $a = 0{\cdot}78$ and $b = 0{\cdot}22$ were determined from the direct data of Expt. 3 as described in the text. These values were substituted in equations (1) and (2) to obtain theoretical curves for f (continuous line) and E (interrupted line), respectively. Semilog. scale.

Comparison of efflux curves using direct and effluent methods

When ^{42}K efflux from a contracting right atrium was followed by counting tissue activity, the semilog. activity–time curve flattened,

becoming linear after about 240 min. For each atrium, effluent data gave a semilog. activity–time curve which was initially steeper than, but finally parallel to, the direct curve (Fig. 3). In fifteen experiments the initial rate constant calculated from effluent data ($\bar{S}_{2(5-25\text{ min})}$) was about 17% (range 12·3–25·7%) greater than that ($\bar{S}_{1(5-25\text{ min})}$) calculated from tissue activity data. The mean difference was highly significant ($P < 0{\cdot}001$). In five of these experiments (Table 3), in which efflux was followed for

TABLE 2. Comparison of methods used for measuring uptake of ^{42}K by atria

Duration of uptake period (min)	No. of expts.		Percentage exchange of atrial potassium (mean ± S.E.)		Significance of difference	
	A	*B*	Successive counting method (*A*)	Single value method (*B*)	*t*	*P*
20	4	3	38·1 ± 1·6	39·9 ± 0·7	0·9	> 0·3
40	11	3	58·9 ± 0·6	61·3 ± 3·1	1·3	> 0·2
80	4	4	89·4 ± 1·2	87·1 ± 1·7	1·1	> 0·3

TABLE 3. Comparison of efflux data obtained by direct and effluent methods

Expt.	Direct $\bar{S}_{1(5-25\text{ min})}$	Effluent $\bar{S}_{2(5-25\text{ min})}$	Direct $\bar{S}_{1(280-360\text{ min})}$	Effluent $\bar{S}_{2(280-360\text{ min})}$	Direct $a:b$	Effluent $ak_1:bk_2$
1	0·0374	0·0400	0·0088	0·0089	0·860:0·140	0·956:0·044
2	0·0297	0·0346	0·0080	0·0074	0·835:0·165	0·962:0·038
3	0·0287	0·0319	0·0093	0·0088	0·780:0·220	0·928:0·072
4	0·0314	0·0395	0·0081	0·0072	0·835:0·165	0·960:0·040
5	0·0317	0·0385	0·0088	0·0092	0·850:0·150	0·963:0·037
Mean	0·0318	0·0369	0·0086	0·0083	0·832:0·168	0·954:0·046

For symbols and dimensions see theoretical section. Slopes were calculated as described in the text. The mean difference between $\bar{S}_{1(5-25\text{ min})}$ and $\bar{S}_{2(5-25\text{ min})}$ is significant ($P < 0{\cdot}01$); that between $\bar{S}_{1(280-360\text{ min})}$ and $\bar{S}_{2(280-360\text{ min})}$ is not significant ($P > 0{\cdot}2$).

more than 300 min, the terminal rate constants ($\bar{S}_{2(280-360\text{ min})}$ and $\bar{S}_{1(280-360\text{ min})}$) obtained by both methods for a given atrium did not differ significantly ($P > 0{\cdot}2$). The atrium of Expt. 3 (the efflux curves of which are illustrated in Fig. 3) was immersed in active saline for 120 min. Potassium exchange at the end of the tracer soak-in period was 94·4%. The other atria were immersed in active saline for only 80 min and their potassium was less fully exchanged (87·1 ± 1·7%). In Table 3 the ratio a/b is shown to be lower in Expt. 3 than in the others, as is expected in a system showing non-homogeneity of potassium exchange.

The direct and effluent curves were adequately described by expressions for a double exponential, such as equations (1) and (2), respectively (Fig. 3). Constants were obtained from tissue activity data as follows. The semilog. slope of the points between 280 and 360 min of efflux (k_2 in equation (1)) was calculated by the method of 'least squares'. The intercept on the f axis of the calculated line drawn through these points gave the ratio of

a/*b*. Counts obtained from this line between 5 and 50 min of efflux were subtracted from total tissue counts at the same times, to give values from which k_1 was calculated by the method of 'least squares'. The four constants obtained were substituted in equations (1) and (2), to obtain data from which theoretical curves were constructed.

DISCUSSION

In certain circumstances direct and effluent methods might measure the activity of different parts of a tissue and give rise to efflux curves which differ in shape. The effluent method gives an average measurement throughout the tissue thickness, while (with a thick tissue and weak radiation) the direct method might indicate what is occurring in the surface layers of the tissue. These layers could conceivably have a different time course of ^{42}K efflux because of a differential distribution of cell sizes with depth. However, the right atrium is a thin tissue (about 0·3 mm thick) and ^{42}K is an isotope which emits strong radiation. Also, measurements throughout the thickness of a single atrium did not reveal a significant difference in cell dimensions between the centre and surface, although there is a thin layer of connective tissue surrounding the cardiac muscle. It is unlikely, therefore, that the above conditions apply. This conclusion is reinforced by the finding that experimental direct and effluent curves bear the relationship to each other predicted in the theoretical section (Fig. 3).

Instead of measuring tissue activity directly, it is possible to obtain an estimate of the direct count by adding together the effluent counts and the final tissue count (cf. Keynes & Swan, 1959). In the present experiments the shape of the 'cumulative' curve is indistinguishable from that obtained by the direct method. Thus, provided that the hypothetical conditions discussed in the previous paragraph do not apply, it is only necessary to measure effluent activity. The particular merits of this technique have been discussed by Hodgkin & Keynes (1955).

In whichever manner the tissue count is obtained, the comparison of early direct and effluent rate constants permits rapid detection of non-homogeneity of ion exchange, before there is obvious departure from simple exponentiality. Provided that early S_2 is larger than early S_1 then simple exponential kinetics are not being followed. In addition to a saving in time (20 min instead of 3–5 hr in the case in point), short experiments minimize net ion change, which itself distorts the efflux curve (Harris & Burn, 1949). These points are well illustrated by reference to the experiments of Keynes & Swan (1959). They showed that the early effluent rate constants of ^{24}Na efflux were consistently larger than the 'cumulative' rate constants (Table 3 of their paper) on transferring labelled frog sartorius muscle to sodium-free lithium Ringer's solution. During short periods of

measurement, tracer efflux did not obviously depart from simple exponential kinetics, although the departure was quite marked late in efflux (Figs. 2 and 4*A* of their paper).

The relationship between direct and effluent curves discussed above follows from the fact that the direct (or 'cumulative') curve *can be described* by the sum of two exponentials. It presumes nothing about the *mechanism* by which the departure from simple exponential kinetics is produced. Some models considered by other authors are discussed below.

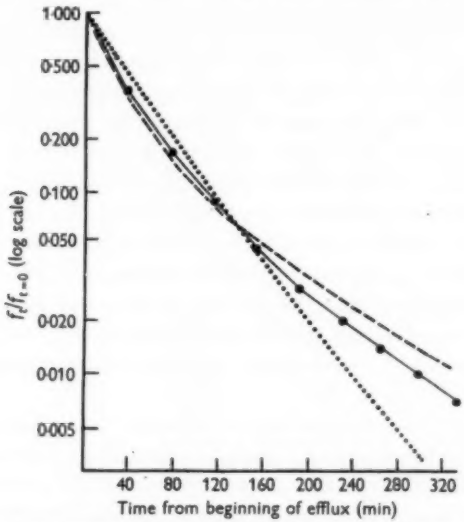

Fig. 4. Direct efflux curve of Expt. 3 (●) compared with some theoretical curves. Dotted line is theoretical curve for model of Harris & Burn (1949) drawn from values obtained by substituting $r = 1{\cdot}1$ in the equation

$$f = \frac{8}{\pi^2}\left[0{\cdot}121\,e^{-k_1 t} + \exp\left(\frac{-k_1 t}{1+r}\right) + 0{\cdot}111\,\exp\left(\frac{-9k_1 t}{9+r}\right)\right].$$

This theoretical curve does not show the degree of bend obtained experimentally. Broken line is theoretical curve for $\lambda = 0{\cdot}6$, according to the model of Creese *et al.* (1956). Agreement with the experimental curve is quite good, but λ estimated from the scatter of cell diameters is only 0·19. Semilog. scale.

Diffusion of ^{42}K through the extracellular fluid causes deviation from simple exponential kinetics (Harris & Burn, 1949). When data obtained in Expt. 3 are applied to the appropriate equations, as used by Creese *et al.* (1956), the value obtained for the constant r is 1·1. On substituting in the relevant equation (Harris & Burn, 1949, p. 520), a semilog. activity–time curve is obtained which only shows a small departure from linearity (Fig. 4). The curvature of the experimental plot is too extensive, therefore, to be explained simply in terms of extracellular diffusion.

Atria are mounted isometrically, contract under tension, and may stretch as experiments progress. The ^{42}K efflux rate constant could be decreased by a continuous enlargement of the extracellular space. However, no deterioration of the mechanical activity of atria is observed during the course of experiments. There is no difference in the percentage exchange of potassium in stretched and unstretched atria, for up to 80 min of immersion in active saline (Persoff, 1959) and cell dimensions do not appear to alter with time.

Creese *et al.* (1956) explained small deviations from simple exponential kinetics in terms of a model in which the scatter of cell diameter sizes produces a log. normal distribution of rate constants. The value of λ (the S.D. of the natural logarithms of the rate constants) giving a curve closest in shape to that of Expt. 3 is 0·6 (Fig. 4). Calculation of the distribution of cell diameters (Fig. 2) gives a λ of 0·19. In Expt. 3 potassium exchange was not quite complete, so that the non-homogeneity was probably a little less than that corresponding to $\lambda = 0{\cdot}6$. Even so, the discrepancy between the two estimates of λ is too great for the scatter of cell diameters to account for the observed deviation from simple exponential kinetics. However, cardiac muscle is a syncytium and variation in the length of fibre between branching points could contribute to the non-homogeneity.

Creese *et al.* (1956) suggested that a further contribution to the non-homogeneity could be provided by variation in the properties of the membranes of different fibres (measured per unit area), independently of variation in fibre diameter. Alternatively, if there were an inverse relation between flux per unit area and fibre diameter, then rate constants would vary as $(\text{diameter})^{-2}$. This would appreciably enhance the apparent non-homogeneity.

Harris & Prankerd (1957) showed that slow intracellular diffusion of an ion would result in departure from simple exponential kinetics. Using the appropriate expressions, they showed that the curve for influx is linear over a large part of its length. If efflux is begun at a time when the influx curve is linear then the efflux curve is linear too, and passes through the origin. The linear parts of both curves are parallel. The data of experiments with two right atria of about the same thickness have been combined to test this model. Tracer uptake by one atrium was followed for a total of 40 min; the other was immersed in active saline for 80 min before measuring efflux by direct counting. Figure 5 illustrates the finding that, while the uptake points lie on approximately the same straight line the efflux points do not, and the model does not account for the shapes of the efflux curves.

Having failed to account completely for the departure from simple

exponential kinetics in terms of some models based on a single compartment, it is necessary to consider the possibility that the atrial potassium is contained within more than one discrete compartment. Both parallel and series arrangements are possible. In terms of a parallel arrangement of two compartments, the fast fraction could be contained within viable cardiac muscle cells. The slow fraction could be contained within the

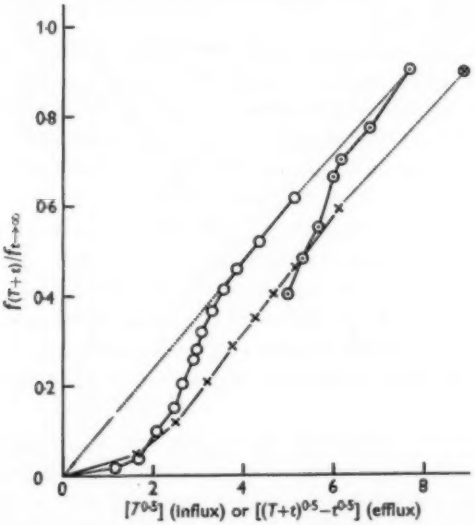

Fig. 5. Influx and efflux data of two experiments with contracting right atria plotted according to the model of Harris & Prankerd (1957). ×, uptake of ^{42}K for 40 min; ○, efflux of ^{42}K from the same atrium after 40 min; ⊗, uptake of ^{42}K at 80 min; ⊙, efflux of ^{42}K from the same atrium after 80 min; T = duration of influx (min); t = duration of efflux (min). Although uptake values of both expts. lie on roughly the same straight line, the efflux curves are not linear. Only the first few efflux values for each expt. lie on the same line, parallel to the uptake line and passing through the origin (dotted line). This model does not account for the deviation from simple exponential kinetics of ^{42}K exchange in the atrium.

connective tissue, which is intimately bound up with, and surrounds, the cardiac muscle. A second possibility relates to the observation that about 10% of the tissue potassium was lost during the period of equilibration. This loss could result from 'death' of, and consequent failure of active transport in, cardiac muscle cells in the centre of the contracting atrium (cf. Creese *et al.* 1958). The slow fraction could be potassium within these 'dead' cells exchanging passively. Either of the alternative possibilities would account for the observation that the non-homogeneity of potassium efflux is greater in contracting than in quiescent atria (Persoff, 1958). This

observation could be explained on the assumption that mechanical activity is associated with a selective increase in the permeability of the viable cardiac muscle membrane to potassium ions.

The simplest series arrangement is that in which the two fractions of potassium are contained within the intracellular and extracellular fluids. However, the extracellular fluid in the atrium contains only about 4 % of the total tissue potassium (Rayner & Weatherall, 1957) and most of it is lost within the first 4 min of efflux. Another series arrangement could be one in which the fast phase of exchange occurs at the cell membrane and the slow phase of exchange takes place at the membrane(s) of some deeply placed intracellular structure(s), for example, the sarcosomes, as was suggested by Rayner & Weatherall (1959).

A useful indirect test for a parallel arrangement of two compartments can be derived from equation (8), which states that if $ak_1 \gg bk_2$, then early $S_2 \doteq -k_1$. In the parallel model k_1 is the rate constant of tracer efflux from the fast fraction. Any alteration in early S_2, produced by an experimental procedure, reflects the change which is occurring in the rate of tracer movement in the fast fraction exclusively. Similarly, it follows from equation (9) *et seq.* that a change in terminal S_2 reflects the change which is occurring solely in the slow fraction. Direct and effluent rate constants ought to be altered to different extents by experimental procedures which affect only one or other of the compartments, in the manner discussed by Keynes & Swan (1959, p. 601). Use could be made of these relationships in experiments with contracting atria, since $ak_1/bk_2 \doteq 24$ (Table 3).

In conclusion, it may well be that all the compartments suggested (and probably others) are present in the atrium. Tracer efflux from more than two compartments can be described *reasonably* well by a double exponential, as was shown by Cohn & Brues (1945). The solution to this particular problem awaits the direct separation of the compartments.

SUMMARY

1. Experiments have been performed in which ^{42}K efflux from a single, contracting, right atrium from the rabbit was measured both by direct-counting and effluent-activity methods. Atrial potassium was in a steady state during the period of measurement of tracer movement.

2. Marked deviation from simple exponential kinetics was observed, as first described by Rayner & Weatherall (1957). The direct efflux curve was accurately described by the sum of two exponentials (Rayner & Weatherall, 1959).

3. In a system in which ^{42}K efflux follows the sum of two exponentials, it has been shown on theoretical grounds that the early rate constant

derived from effluent data is larger than that derived from direct data. The late rate constants are the same by both methods.

4. These criteria for heterogeneity of efflux apply satisfactorily to experiments with atria. The early rate constants derived from effluent data were about 17 % (mean) larger than those derived from direct data, while the late rate constants were equal.

I am greatly indebted to Professor Miles Weatherall for making freely available the facilities of his laboratory, for his constant supervision and advice, and for giving me access to his histological preparations of right atria. It is a pleasure to acknowledge, with many thanks, the time spent by Dr R. Creese in helpful discussion. I am indebted to Mr J. O'Kelly and Miss S. Gross for technical assistance and to Mr J. E. Linder for the histological preparations. The expenses of this research were met by a scholarship awarded by the Governors of the London Hospital Medical College.

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INTRACELLULAR HYDROLYSIS OF DIPEPTIDES DURING INTESTINAL ABSORPTION

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Newey & Smyth (1959*a*) found that when solutions containing certain dipeptides were placed in contact with the intestinal mucosa these were not transported as such, but the constituent amino acids were found on the other side of the intestinal barrier, i.e. *in vitro* in the fluid bathing the serosal surface, or *in vivo* in the blood stream. The only exception was glycyl-glycine, when it was found that small amounts of the dipeptide passed through the mucosal barrier, but even here the amount of dipeptide transported was small, and by far the greatest amount was transported as glycine. The *in vitro* results have been confirmed for other dipeptides by Wiggans & Johnston (1959). The present experiments were undertaken to investigate the site of dipeptide hydrolysis and in particular to determine whether dipeptides could enter the mucosal cells. A preliminary account has been given by Newey & Smyth (1959*b*).

METHODS

In vitro. The technique was that of the sac of everted small intestine of Wilson & Wiseman (1954). Rats were anaesthetized with pentobarbitone sodium, and the small intestine removed below the level of the duodenum. This was everted and divided into five approximately equal lengths, and sacs were made from one or other of these lengths. The sacs contained 1 ml. bicarbonate saline (Krebs & Henseleit, 1932) and were suspended in 50 ml. bicarbonate saline in equilibrium with 5 % CO_2 and 95 % O_2 in flasks, as described by Parsons, Smyth & Taylor (1958), and shaken at 80 cycles/min in a Warburg bath at 38° C. In all cases the saline contained initially 500 mg glucose/100 ml. saline. Peptides were added as described in subsequent sections.

The principle used in deciding whether peptide hydrolysis occurred in the fluid in which the sacs were suspended, or whether it occurred after peptide entered the cells, was as follows. If sacs are incubated with a glycyl peptide present in the outer (mucosal) solution only, it is found that at the end of 1 hr both mucosal and serosal fluids contain considerable amounts of glycine. This formation of glycine could be due to (1) peptidases present in the mucosal fluid, (2) peptidases inside the mucosal cells acting on peptides which had entered these cells, or (3) peptidases in the serosal fluid acting on peptides which had escaped intracellular hydrolysis and passed unchanged into the serosal fluid. Hydrolysis in the last two sites can occur only if peptides enter the mucosal cells from the mucosal fluid. Hence the difference between the total peptidase activity of the system and the activity due to peptidases in the mucosal fluid will be a measure of the entry of peptides into the mucosal

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cells. The total peptidase activity was estimated as follows. A sac of everted intestine was made and suspended in mucosal fluid containing peptide, and incubated for 30 min. The sac was then removed, opened and the serosal fluid drained out. To this was added an extract of the intestine, and the glycine content of this mixture determined. A sample of mucosal fluid was taken, the glycine content of this determined and from this the total glycine in the mucosal fluid was calculated. This, added to the glycine in the combined serosal fluid and intestinal extract, gave the total peptidase activity.

As a measure of the peptidase activity of the mucosal fluid it was not permissible to take the amount of glycine present in the mucosal fluid at the end of the incubation period, because (*a*) some glycine produced by peptidases in the mucosal fluid might be taken up by the mucosal cells, and (*b*) some glycine produced in the mucosal cells might diffuse back into the mucosal fluid. (The results presented show that this did in fact occur.) Instead, the following procedure was used. Preliminary experiments showed how much peptide disappeared from the mucosal fluid during the 30 min incubation. After collecting the mucosal fluid at the end of this period and after taking a sample, an amount of peptide was added to the mucosal fluid to replace approximately that likely to have disappeared. The mucosal fluid was then incubated for a second period of 30 min, and another sample taken for estimation. The increase in glycine content during this second incubation period was a measure of the peptidase activity in the mucosal fluid. This involved, however, the assumption that the peptidase activity was stable during incubation. To control this, the mucosal fluid was incubated for a third 30 min period. Comparison of the glycine produced in the second and third periods was an indication of the stability of the peptidase. Further control experiments were carried out as described later.

In describing the results certain terms are used which should be clearly defined. The term 'first incubation period' is used to refer to the period when the fluid was being incubated in the presence of the sac of intestine, the 'second incubation period' and 'third incubation period' refer to the subsequent incubations after the sac had been removed. 'Total peptidase activity' is used to refer to the activity of all the peptidases present in the mucosal fluid, intestinal wall and serosal fluid, and these peptidases are referred to collectively as 'total peptidases'. The term 'mucosal-fluid peptidase activity' or 'mucosal-fluid hydrolysis' is used to refer to the hydrolysis of peptides by the fluid in which the sacs are suspended. It is also convenient to have a term to refer to the hydrolysis which occurs after peptides have entered the mucosal cells. This term must include hydrolysis occurring both in the wall of the intestine and inside the sac. For this it is proposed to use the term 'intraintestinal hydrolysis'. No better term suggests itself, and although not strictly accurate it avoids repetition of a much longer phrase. It is in fact quite immaterial to our argument whether 'intraintestinal hydrolysis' takes place in the wall of the intestine or inside the sac, and Newey & Smyth (1959*a*) have produced evidence suggesting that the peptidase activity of the serosal fluid is small compared with the total peptidase activity.

In vivo. Rats were anaesthetized with pentobarbitone sodium and the procedure used was that described by Sheff & Smyth (1955). If saline containing peptide is circulated through a loop of intestine it is found that peptide disappears. This loss of peptide from the luminal fluid could be due to (1) hydrolysis of peptide by peptidases in the luminal fluid or (2) entry of peptides as such into the mucosal cells. The difference between the total amount of peptide which disappears and the amount hydrolysed by peptidases in the luminal fluid will be a measure of the amount of peptide which enters the mucosal cells. In order to determine the total amount of peptide which disappears, 50 ml. of solution containing peptide was circulated for 30 min. At the end of this period the amount of peptide remaining was estimated and hence the amount which disappeared was calculated. In order to determine the amount of hydrolysis due to peptidases in the luminal fluid, saline containing no peptide was circulated through the intestine of the rat for 30 min. At the end of this period the fluid was collected, peptide was added to make the desired concentra-

tion, and the solution was incubated for a further 30 min and the extent of hydrolysis determined.

Chemical. The peptides used were glycyl-glycine, glycyl-L-tyrosine, glycyl-L-leucine, L-leucyl-glycine and glycyl-L-tryptophan. These were obtained commercially. They were tested for the presence of free glycine both before and after acid hydrolysis. No significant amounts of free glycine were present in any of the peptides. The determination of peptide was carried out by estimation of free and bound glycine by the same methods as used previously by Newey & Smyth (1959*a*).

RESULTS

Experiments in vitro

The results can be illustrated with an experiment on glycyl-glycine, which is included in Table 1. A sac was made from the middle fifth of the intestine. This was filled with 1 ml. of bicarbonate saline containing 500 mg glucose/100 ml. It was suspended in 50 ml. of the same solution containing in addition glycyl-glycine in concentration 0·0151 M, and incubated in aerobic conditions. After 30 min the sac was removed and the contents drained out and added to an extract of the sac. The combined solution was subsequently found to contain 103 μmole of glycine. The mucosal fluid contained 88 μmole of glycine, and hence the total peptidase activity of the system produced 191 μmole of glycine. To the mucosal fluid was now added 151 μmole of glycyl-glycine, as preliminary experiments had shown that this would bring the peptide content roughly to its initial level. On incubation of the mucosal fluid for 30 min the increase in glycine was 8·0 μmole. On further incubation for a third period of 30 min the increase in glycine concentration was 6 μmole. The glycine production during the second period (8 μmole) is that produced by peptidase in the mucosal fluid. This is very much smaller than the total glycine produced during the first period (191 μmole) and it therefore follows that most of the glycine production during the first period was due to intraintestinal peptidases, which includes also possible peptidase activity at the mucosal surface of the cells. The difference between the production in the second and third periods represents deterioration of the peptidase during incubation, and the small change in glycine production between the second and third periods makes it unlikely that the results could be explained by a very large deterioration of the mucosal fluid peptidases during the first period.

This control experiment on stability of peptidase is not completely satisfactory, as it is just possible that there may be a rapid deterioration during the first incubation period, which is not indicated by the difference between the second and third incubation periods. Furthermore, the amounts of glycine produced by the mucosal fluid peptidases during the second and third periods are small, and hence the error in the measurement

is relatively large. A further control experiment was therefore done in which a section of gut was homogenized in 110 ml. bicarbonate saline in order to get a solution containing peptidase without having to incubate for 30 min. Two 50 ml. fractions were taken: to one was added 2272 μmole glycyl-glycine, and both were incubated for 60 min at 38° C. To the one which had contained no glycyl-glycine 2272 μmole was now added and this was incubated for a further 60 min. The glycine content of the two fractions was determined; the first was found to contain 790 μmole and the second 750 μmole. This shows that there was no substantial deterioration of peptidase activity during the first 60 min incubation.

TABLE 1. Production of glycine from various dipeptides by mucosal fluid peptidases and total peptidases of sacs of intestine. The sacs contained 1 ml. bicarbonate saline with glucose, and were suspended in 50 ml. bicarbonate saline containing glucose and dipeptide. The initial concentration of dipeptide in all cases was 0·0151 M. The duration of the 1st, 2nd and 3rd incubation periods was in all cases 30 min. For explanation of table, see text

	Glycine produced by total peptidases (1st incubation period) (μmole)			Glycine produced by mucosal fluid peptidases (μmole)	
Dipeptide	Mucosal fluid	Serosal fluid and extract of intestine	Total	2nd incubation period	3rd incubation period
Glycyl-glycine	88	103	191	8	6
	72	89	161	8	16
Glycyl-L-leucine	134	73	207	93	70
	110	75	185	98	60
Glycyl-L-tyrosine	55	79	134	30	28
	68	67	135	50	38
L-leucyl-glycine	113	97	210	65	62
	104	89	193	60	56
Glycyl-L-tryptophan	110	37	147	53	39
	68	42	110	42	45

The experimental conditions actually minimize the difference between total peptidase activity and mucosal fluid peptidase activity, for the following reason. During the second period of incubation the peptidase activity of the mucosal fluid was due to peptidases which had accumulated during the whole of the first period of incubation, and therefore the peptidase activity of the mucosal fluid during the first incubation period was probably less than that estimated during the second incubation period. This over-estimation of the peptidase activity in the first period will be more important with those peptides where the mucosal fluid peptidase activity is much higher than is the case with glycyl-glycine.

It will be seen that during the first incubation period 88 μmole of glycine accumulated in the mucosal fluid, whereas only a very small quantity of this could have been produced by the mucosal fluid peptidases. This means

either that peptidase activity must have occurred at the mucosal surface of the cells, or alternatively that after intraintestinal hydrolysis some of the glycine formed diffused out of the cell back into the mucosal fluid.

The results of a number of similar experiments with other dipeptides are included in Table 1. In this table the third column of figures gives the glycine produced by the total peptidase activity of the sac of intestine, the fourth column gives the activity of the mucosal fluid peptidases, and hence the difference between these indicates how much of the total peptidase activity is accounted for by intraintestinal peptidases. In all cases it is seen that at least 50 % of the total peptidase activity must be accounted for by intraintestinal peptidases. Only in the case of glycyl-leucine is the figure as low as 50 % and in most cases it is much greater. In the case of glycyl-glycine almost all the activity is due to intraintestinal peptidases. The difference between the last two columns indicates the extent of deterioration of the peptidase during incubation. The magnitude of this difference is relatively small, and certainly does not suggest that the difference between peptide hydrolysed during the first and second periods can be explained by deterioration of the peptidase during incubation.

It is also seen that in all cases there is a considerable amount of glycine in the mucosal fluid at the end of the first incubation period. This shows, as already suggested in the case of glycyl-glycine, that either glycine diffuses back into the mucosal fluid after intraintestinal hydrolysis, or alternatively that hydrolysis takes place at the surface of the mucosal cells, with liberation of glycine into the mucosal fluid.

Experiments in vivo

The results of the experiments *in vivo* are shown in Tables 2 and 3. Table 2 gives the results of experiments where solutions of dipeptide were circulated through the intestine and the total amount of peptide disappearance and hydrolysis of peptide determined. In this table the 'peptide disappeared' means the difference between the peptide added and the peptide recovered from the luminal fluid. It includes therefore peptide accounted for as glycine in the luminal fluid at the end of the experiment. 'Peptide absorbed' is the difference between the peptide disappeared and the peptide accounted for as glycine in the luminal fluid at the end of the experiment. In interpreting the table it should be remembered that 1 mole of glycyl-glycine gives 2 moles of glycine on hydrolysis, whereas 1 mole of the other peptides gives only 1 mole of glycine. Table 3 gives the results of experiments where the peptidase activity of the luminal fluid was determined. The argument can best be followed by reference to an example. Glycyl-leucine in initial concentration 0·0378 M was circulated through the intestine for 30 min, the initial amount of peptide present

being 1890 μmole (Table 2). After 30 min the luminal fluid contained 151 μmole glycine and 925 μmole glycyl-L-leucine. Since 1890 μmole peptide was present initially, 965 μmole had disappeared. Of this, 151 μmole was accounted for by glycine in the luminal fluid, and hence

TABLE 2. Disappearance of dipeptides from the rat intestine *in vivo*. The peptide was present initially in 50 ml. 0·9% NaCl solution in the concentration shown. The peptide absorbed is the amount not accounted for at the end of the experiment, either as glycine or peptide; the peptide disappeared is the amount absorbed + the amount accounted for as glycine in the luminal fluid

Peptide	Initial concn. (M)	Duration of expt. (min)	Initial peptide (μmole)	Final amount in luminal fluid: Glycine (μmole)	Final amount in luminal fluid: Peptide (μmole)	Peptide absorbed (μmole)	Peptide disappeared (μmole)
Glycyl-glycine	0·0378	30	1890	164	980	828	910
	0·0378	30	1890	258	1081	680	809
	0·0378	60	1890	168	337	1469	1553
	0·0378	60	1890	224	208	1570	1682
Glycyl-L-leucine	0·0378	30	1890	151	925	814	965
	0·0378	30	1890	609	609	672	1281
	0·0531	60	2655	302	915	1438	1740
	0·0266	60	1330	160	58	1112	1272
Glycyl-L-tyrosine	0·0378	30	1890	179	722	989	1168
	0·0378	30	1890	205	835	850	1055
L-leucyl-glycine	0·0378	30	1890	153	770	967	1120
Glycyl-L-tryptophan	0·0151	30	755	198	349	208	406

TABLE 3. Hydrolysis of dipeptides by luminal fluid peptidases. The peptides in the initial concentration shown were incubated 30 or 60 min in 0·9% NaCl solution which had been previously circulated through the intestine for an equal time interval

Peptide	Initial concn. (M)	Period of incubation (min)	Peptide hydrolysed (μmole)
Glycyl-glycine	0·0378	30	60
	0·0378	30	38
	0·0378	60	142
	0·0378	60	169
Glycyl-L-leucine	0·0378	30	144
	0·0378	30	346
	0·0531	60	479
	0·0266	60	324
Glycyl-L-tyrosine	0·0378	30	145
	0·0378	30	179
L-leucyl-glycine	0·0378	30	92
	0·0378	30	103
Glycyl-L-tryptophan	0·0151	30	210

814 μmole had been absorbed. This absorption could have taken place either as peptide or as amino acid after hydrolysis of the peptide in the lumen of the intestine by luminal peptidases. It is possible to decide between these two possibilities by examination of the corresponding experiments

in Table 3, i.e. those where the same concentration of peptide was incubated for 30 min with luminal fluid. In the experiments given in Table 3 the total hydrolysis of glycyl-L-leucine by luminal fluid peptidases was 144 μmole and 346 μmole, respectively. Even the larger of these figures is far less than the total peptide which disappeared when circulated through the intestinal lumen, 965 μmole. The only possible conclusions are that glycyl-L-leucine entered the mucosal cells as such, or at least that it was hydrolysed at the surface of the cells. The results are certainly incompatible with the hydrolysis of peptide by luminal fluid peptidases before its absorption. It should also be remembered, as was pointed out in the case of experiments *in vitro*, that the technique minimizes the difference between luminal fluid peptidase activity and disappearance of peptide, since the experiments for estimating luminal fluid peptidase activity were carried out with fluid in which the peptidases had accumulated for 30 min, whereas in the experiment where peptide was present initially in the intestine the average peptidase activity must have been considerably smaller.

Examination of Tables 2 and 3 shows that a similar conclusion could be drawn for all the dipeptides used, i.e. that the dipeptide can enter the mucosal cell without being hydrolysed in the lumen of the intestine, or at least that hydrolysis takes place on the surface of the cell and not in the luminal fluid. Just as in the case of the experiments *in vitro*, there is a considerable amount of glycine present in the luminal fluid at the end of the experiment. In the case of glycyl-glycine, this is much larger than the amount which could be produced by luminal fluid peptidases, which would therefore imply that, after intracellular hydrolysis, some glycine diffuses back again into the lumen of the intestine. In the case of the other dipeptides the amount in the lumen is of the same order as the amount which could be produced by luminal fluid peptidases.

Back diffusion of glycine

It has been suggested above that the high concentration of glycine in the mucosal fluid after incubation of peptide with sacs of intestine is due to back diffusion of glycine. Wiseman (1955) showed that glycine was moved by the hamster intestine *in vitro* against a concentration gradient, the final concentration in the serosal fluid being 1·7 times the final concentration in the mucosal fluid. The precise value of the ratio will depend on the concentrations used, but it is necessary to show that the glycine concentration in the final serosal fluid is considerably greater than that in the final mucosal fluid in order to produce conditions favouring back diffusion of glycine. It was not possible to calculate accurately from the data the concentrations of glycine in the final mucosal and serosal fluid, as

the volumes were not always measured. It was, however, possible to calculate the approximate concentrations by taking values for the volumes obtained in analogous experiments. Figures were taken to give the maximum possible mucosal concentration and the minimum possible serosal concentration, so that the figures calculated for concentration gradient of glycine between final mucosal and serosal fluid would be minimum figures. The following results were obtained: for glycyl-glycine final mucosal concentration of glycine = 0·0018 M, final serosal concentration = 0·03 M; for glycyl-L-leucine final mucosal concentration of glycine = 0·0025 M, final serosal concentration = 0·013 M; for glycyl-L-tyrosine the final mucosal concentration of glycine = 0·0013 M, final serosal concentration = 0·013 M; for L-leucyl-glycine final mucosal concentration of glycine = 0·002 M, final serosal concentration = 0·017 M; for glycyl-L-tryptophan final mucosal concentration = 0·002 M, final serosal concentration = 0·008 M. There is therefore little doubt that the final concentration of glycine on the serosal side of the epithelial cell is much greater than on the mucosal side, and this could probably account for back diffusion of glycine.

In order to show directly that back diffusion of glycine occurred the following experiments were carried out. A sac of intestine was incubated for 30 min with 0·0151 M glycyl-glycine. The sac was then withdrawn, rinsed with warm saline, and returned to a flask containing 50 ml. bicarbonate saline with glucose but without peptide or glycine and incubated a further 30 min. At the end of this period the mucosal fluid was found to contain 36 μmole of glycine. In another experiment a sac of intestine contained initially 2 ml. of bicarbonate-saline containing 0·066 M glycine. This was incubated in 50 ml. of bicarbonate-saline containing no glycine or peptide. At the end of 30 min the mucosal fluid contained 20 μmole of glycine.

These two experiments show clearly that glycine can diffuse back across the intestine into the mucosal fluid. Although in both cases the amount of glycine diffusing back is smaller than the amount of glycine found in the mucosal fluid when sacs were incubated with peptide in the mucosal fluid, this is hardly surprising, as the latter conditions would be much more favourable to maintaining a high concentration of glycine in the mucosal cells than in either of the two experiments just described.

Total peptidase activity of the intestine

It was of some interest to compare the peptidase activity obtained under the experimental conditions described with the peptidase activity which can be obtained by making homogenates of the intestine. Fifths of intestine were homogenized in 50 ml. of bicarbonate saline; to this homo-

genate was added peptide to make the required concentration, the flasks were incubated for varying periods and the amount of hydrolysis determined. The results of experiments with glycyl-glycine and glycyl-L-leucine are seen in Table 4. In the first experiment with glycyl-L-leucine nearly 85 % of the peptide was hydrolysed in 30 min and therefore an experiment of only 15 min duration was also carried out. Even in this case there was hydrolysis of about 75 % of the peptide. Table 4 clearly shows that the extent of hydrolysis in the homogenates is very much greater than in the sac experiments, and also much greater than would be necessary to account for the hydrolysis of the peptide which disappears from the intestine *in vivo*. The activity of the homogenates is more striking when one considers that the enzymes and necessary co-enzymes or activators are diluted in a volume of 50 ml. instead of the volume (approximately

TABLE 4. Hydrolysis of peptide by homogenates of intestine. The 1st, 3rd and 5th fifths of the jejunum and ileum were homogenized in bicarbonate saline containing 500 mg glucose/100 ml. The initial concentration of peptide present in all cases was 0·0378 M, and the initial amount 1890 μmole. In the first four experiments in the table the intestine was homogenized in 50 ml. of saline. In the last experiment a similar homogenate was diluted 10 times with saline

Peptide	Period of hydrolysis (min)	Peptide hydrolysed (μmole)		
		1st sac	3rd sac	5th sac
Glycyl-glycine	30	271	306	360
Glycyl-glycine	30	283	315	400
Glycyl-L-leucine	30	1600	1605	1630
Glycyl-L-leucine	15	1417	1417	1515
Glycyl-L-leucine	15	426	573	394

1 ml.) of the segment of intestine. The capacity for intracellular hydrolysis in physiological conditions might therefore be very much greater than is indicated by these figures. It is also seen from Table 4 that the capacity of the intestine to hydrolyse glycyl-L-leucine is much greater than its capacity to hydrolyse glycyl-glycine.

Site of peptidase activity

While the experiments quoted above show that there is much more peptidase activity inside the cells than on the mucosal surface, they do not prove that hydrolysis of peptide initially present in the mucosal fluid does not take place at the cell surface, and experiments were carried out to test this point specifically. Before describing these it is, however, necessary to define more precisely what is meant by hydrolysis at the mucosal surface of the cell and intracellular hydrolysis. By hydrolysis at the surface, we mean that peptides are hydrolysed at a site on the cell membrane, the constituent amino acids are set free quantitatively in the

luminal or mucosal fluid, and transfer of these by the intestine is indistinguishable from that of amino acids initially added to the mucosal fluid. By intracellular hydrolysis we mean that the site of hydrolysis is such that the amino acids are not set free quantitatively in the mucosal fluid, but are set free in the cell, although some of them may later diffuse back into the mucosal fluid from the interior of the cell.

The principle of these experiments was as follows. Sacs of intestine were incubated with glycyl-glycine, and the final amount of glycine determined in the mucosal fluid, the intestinal wall and the fluid inside the

TABLE 5. Comparison of the final amounts of glycine in the mucosal fluid, the intestinal wall and the serosal fluid, when sacs of intestine were incubated for 30 min in fluid containing 0·0151 M glycyl-glycine and 0·0046 M glycine, respectively. The sacs contained 1 ml. bicarbonate saline with glucose, and were suspended in 50 ml. bicarbonate saline containing glucose and either glycyl-glycine or glycine. For explanation of concentrations used see text

Initial mucosal fluid	Final glycine content (μmole)			Amount of glycyl-glycine hydrolysed (μmole)
	Mucosal fluid	Intestine	Serosal fluid	
Glycyl-glycine 0·0151 M	88	51	52	96
	72	49	40	81
	98	58	69	113
	92	41	52	93
	89	51	68	104
	67	71	72	105
Mean	84	54	59	99
Glycine 0·0046 M	193	19	19	—
	201	26	26	—
	199	27	23	—
Mean	198	24	23	—

sac (serosal fluid). The total amount of glycine formed could therefore be calculated. If hydrolysis took place at the mucosal surface of the cell, this total amount of glycine must have been returned to the mucosal fluid during the course of the experiment and then have been transferred by the intestine, so that ultimately part of it appeared in the intestinal wall and part in the serosal fluid. Another set of experiments was now done in which the total amount of glycine formed in the first set of experiments was put into the mucosal fluid initially, and the final amounts of glycine determined in the intestinal wall and in the serosal fluid. These values were compared with those in the first set of experiments when glycyl-glycine was present initially in the mucosal fluid. The results are given in Table 5. When glycyl-glycine (0·0151 M) was present initially in the mucosal fluid the average amount hydrolysed was 99 μmole, and the maximum amount in any one experiment was 113 μmole. Hence the maximum amount of glycine formed was 226 μmole. In the second set of experiments glycine was initially present in a concentration of 0·0046 M.

This is equivalent to 230 μmole of glycine in the mucosal fluid, an amount greater than the maximum amount formed in the first set of experiments. It is therefore the maximum amount of glycine which could have been present in the mucosal fluid in the first set of experiments if hydrolysis took place at the cell surface, and also if it took place instantaneously so that the total amount produced was present at one time. Actually the glycine concentration in the mucosal fluid in the first set of experiments would probably be considerably smaller than this. In these conditions it is seen that the final amounts of glycine in the intestinal wall and in the serosal fluid were very much smaller than those in the first set of experiments when glycyl-glycine was initially present. These results leave little doubt that hydrolysis of glycine cannot take place exclusively at the surface of the cell, and that at least part of it must occur intracellularly.

DISCUSSION

The results presented show that when solutions of various peptides are in contact with the rat intestinal mucosa a much greater amount of hydrolysis occurs than could be accounted for by the peptidase activity of the mucosal fluid. The only conclusions possible would appear to be either (*a*) that peptide enters the mucosal cell as such, or (*b*) that hydrolysis takes place at a site on the cell membrane. While some hydrolysis at the cell membrane may take place, this cannot explain the results presented in Table 5, and the conclusion can be drawn that at least some of the dipeptide is hydrolysed intracellularly. This view is supported by the fact that peptidase activity is very much greater in homogenates of intestine than the activity observed in the everted sacs or *in vivo*, a finding which can be explained by assuming that most of the peptidases are inside the cell and not on the surface. The results of Holter (1954) on intracellular distribution of enzymes have some bearing on the matter, as he found in *amoeba* that peptidases, unlike some of the other enzymes, are not associated with any centrifugable structure, but appear to be distributed throughout the cell cytoplasm. All of these points are against peptidase activity being exerted at the mucosal surface of the cell membrane.

Newey & Smyth (1959*a*), in discussing the problem of the form in which proteins are absorbed, pointed out that there is not one problem but two problems, i.e. the form in which proteins enter the mucosal cells from the lumen of the intestine, and the form in which they enter the blood stream from the mucosal cells. Their results offered some evidence relative to the second question, i.e. how proteins enter the blood stream, and they showed that when dipeptides are present in the lumen of the intestine it is mainly amino acids and not dipeptides which emerge from the serosal side of the epithelial cells. Their results with dipeptides *in vitro* were

confirmed by Wiggans & Johnston (1959). We do not know of any substantial evidence that proteins, in amounts of nutritional significance, enter the blood stream of the adult animal in any form other than amino acids. The present results offer some answer to the first question posed by Newey & Smyth (1959*a*), in what form can proteins enter the mucosal cells from the lumen of the intestine? The answer is quite clear that dipeptides can enter the mucosal cells without preliminary hydrolysis to amino acids in the lumen of the intestine. Further experiments by a similar technique, with larger amino-acid aggregates, would presumably answer the question what size of protein derivative can enter the mucosal cells from the intestinal lumen. Since completing this work we became aware of the paper by Cajori (1933) in which a very similar idea to that which we have used was applied to the absorption of peptone by the Thiry–Vella loop in dogs. Cajori was rather hesitant about drawing any very definite conclusions from his results, but to us they would suggest that peptone could enter the mucosal cells without preliminary hydrolysis. The term peptone of course is rather a vague term without definite indication of the size of molecule involved, but it would seem possible that molecules larger than dipeptides can enter the mucosal cells. It must therefore be envisaged that a possible route for protein absorption is partial hydrolysis in the lumen of the intestine to peptides of various sizes, entry of these into the mucosal cells, intracellular hydrolysis to amino acids and passage of the amino acids into the blood stream.

Of the dipeptides studied glycyl-glycine seems to be the one most likely to escape hydrolysis in passage through the mucosal cells, and small quantities have been shown to pass through the intestine by Agar, Hird & Sidhu (1953), Newey & Smyth (1959*a*) and Wiggans & Johnston (1959). A possible explanation of this, in keeping with the present results, is that the intestine possesses a smaller capacity for hydrolysis of glycyl-glycine than for the other dipeptides. This may be because glycyl-glycine is formed during digestion in the intestine only in very small quantities, and hence mechanisms for dealing with it have not been evolved. Our knowledge of the amino-acid sequences in various proteins is still very limited, and clearly the possible dipeptides which could be formed during intestinal digestion must depend on the amino-acid sequences in the protein. Two consecutive glycine molecules have been found by Smith, Hill & Kimmel (1958) in papain, which also contains three consecutive alanine molecules. Consecutive appearances of the same amino acid are on the whole not a prominent feature of the known peptide structures, and even if they occur would not necessarily result in the formation of the particular dipeptide containing both the amino-acid molecules, so that glycyl-glycine is unlikely to be produced in large amounts. It must be admitted, however,

that little is known yet of the amino-acid sequences in the dietary proteins, and only when these are known will it be possible to see how the specificity of the digestive enzymes is related to the naturally occurring peptides.

A number of older workers (see review by Starling, 1906) believed that the activity of the succus entericus was largely due to the shedding of epithelial cells into the intestinal lumen with liberation of the contained enzymes, and the view was even put forward, although without much evidence, that hydrolysis of a number of food substances occurred actually during absorption. Our results supply evidence to show that there was probably a considerable amount of truth in these older views, at least so far as certain dipeptides are concerned.

SUMMARY

1. A method is described for comparing, both *in vivo* and *in vitro*, the peptidase activity of the fluid in contact with the intestinal epithelium and the total amount of dipeptide which disappears from the fluid.

2. It was found that with all the dipeptides tested, glycyl-glycine, glycyl-L-leucine, glycyl-L-tryptophan, glycyl-L-tyrosine and L-leucyl-glycine, the amount of dipeptide which disappears from the mucosal fluid could not be accounted for by the peptidase activity of the mucosal fluid.

3. After absorption of glycyl-glycine the amount of glycine present in the intestinal wall and serosal fluid was greater than could be accounted for by hydrolysis at the cell membrane and subsequent absorption of glycine.

4. The peptidase activity of homogenates is considerably greater than that of the intact absorbing intestine.

5. It is concluded that the dipeptides are able to enter the mucosal cells without previous hydrolysis.

6. The relation of these findings to protein absorption is discussed.

We are grateful to Mr I. G. Dart for excellent technical assistance. We also wish to acknowledge financial support from the Medical Research Council and from John Wyeth and Brother Ltd.

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OXYTOCIC ACTIVITY OF RABBIT'S BLOOD APPLIED DIRECTLY TO SUPERFUSED RAT UTERUS

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(*Received* 4 *March* 1960)

The detection and measurement of oxytocin in blood presents certain difficulties. It has usually been thought necessary to make an extract of blood before assaying it on the isolated rat uterus, but the methods of extraction available are laborious and not altogether satisfactory (Bisset & Walker, 1954; Bisset & Lee, 1957; Ginsburg & Smith, 1959; Walker, 1960). In the first place, there is a variable loss of activity during extraction. Secondly, the presences of non-specific oxytocic substances in the extracts interferes with the assay; these substances are more likely to be present if the blood is concentrated during extraction, and this usually has to be done if the very small amounts of oxytocin normally present in blood are to be detected.

We have tried to overcome some of these difficulties by applying blood from a rabbit directly to a sensitive preparation, the 'superfused' rat uterus previously soaked in dibenamine (*N*:*N*-dibenzyl-β-chloroethylamine). Blood applied in this way is without activity. If, however, oxytocin is infused intravenously into the donor animal, samples of blood tested on the uterus become active, and the concentration of oxytocin in them can be measured approximately. In this way the rate of inactivation of the infused oxytocin can be estimated, and factors affecting its inactivation can be studied. If now the rate of infusion is reduced until samples of blood regularly possess only just detectable activity, any extra activity, resulting from release of oxytocin in the animal after certain stimuli, is easily demonstrated by the increased size of the contractions of the rat uterus.

By this method a detectable increase in the oxytocic activity of the blood can be brought about by intracarotid injections of 3% NaCl, hot 0·9% NaCl, or nicotine, and by stimulation of the central end of the left vagus. The uterus, having been soaked in dibenamine, was insensitive to 5-hydroxytryptamine, and it is therefore likely that the responses were caused by oxytocin itself, though there is no proof of this.

METHODS

Rabbits of either sex, weighing 1·5–2·5 kg, were lightly anaesthetized with 25 % (w/v) urethane given intravenously. A polythene tube of 1·5 mm diameter (*A* in Fig. 1) was filled with saline (0·9 % NaCl) solution and inserted into the left common carotid artery towards the heart. This tube was led to a three-way Perspex tap *B*, which in turn was connected by polythene tubing to a similar tap *C*. When taps *B* and *C* were turned to the appropriate positions and the plunger of the syringe was withdrawn, blood flowed from the rabbit into the tubing almost as far as tap *C*. Tap *B* was then turned so that pressing the plunger of the syringe expelled blood on to the superfused uterus. When tap *B* was turned back to its original position the rest of the blood could be pushed back into the rabbit. Each time blood was tested the rabbit lost very little more than the volume of the sample, which was 0·5 ml. Tap *C* was connected to a beaker of saline solution so that the syringe could be refilled if necessary.

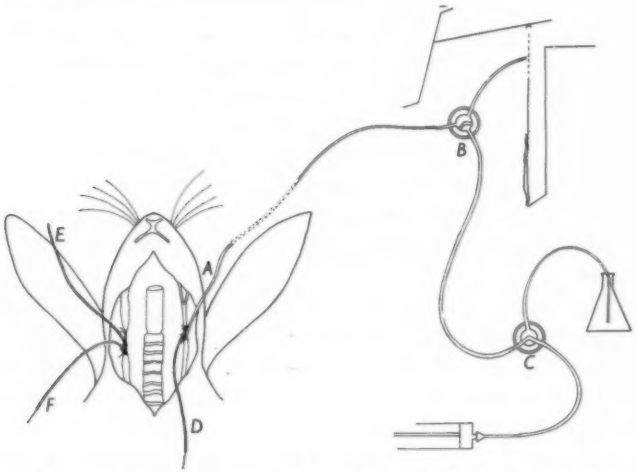

Fig. 1. Diagram showing method of applying rabbit's blood directly to the superfused rat uterus. For details see text.

Intracarotid injections (0·25–1·0 ml.) were given through a polythene tube *D*, 1·0 mm in diameter, which was inserted into the left common carotid artery towards the head. The injections were given rapidly, at about 1 ml./sec, except where specially mentioned. A tube *E*, also 1·0 mm in diameter, was tied into the right external jugular vein and solutions were infused into it from a Palmer Slow Infusion Apparatus. In some experiments another fine tube *F* was also inserted into the right external jugular vein to make possible more rapid intravenous injections. All tubes were of polythene, so as to avoid the possible production of kinin-like oxytocic substances which form when the blood of some species comes in contact with glass (Armstrong, Jepson, Keele & Stewart, 1955). The rabbit received 20–25 mg heparin intravenously before the tubes were inserted.

The uterus of the virgin rat in oestrus was used as test preparation. After being soaked for 30 min in de Jalon's solution containing dibenamine 100 μg/ml., it was superfused with de Jalon's solution at 27–28° C (Gaddum, 1953). The preparation usually responded to solutions of oxytocin 0·05–0·1 m-u./ml. Samples of 0·5 ml. blood were applied at 5 min intervals to the surface of the uterus and allowed to remain in contact with it for 30 sec

before superfusion was restarted. A vibrator (Bülbring, Crema & Saxby, 1958), attached to the rod on which the lever was mounted, prevented the writing point from sticking to the surface of the drum.

The vagus was stimulated with square-wave pulses of 5 msec duration, at a frequency of 10/sec for a period of 15–20 sec.

Pitocin (Parke, Davies) was used as preparation of oxytocin.

The method was demonstrated to the Physiological Society and has been briefly described (Siddiqi & Walker, 1959).

Fig. 2. Superfused rat uterus. ○ Application of oxytocin solutions. In this and all subsequent figures ● indicates applications of rabbit's arterial blood at 5 min intervals.

RESULTS

Application of arterial blood to the superfused uterus

Samples of carotid blood taken from the anaesthetized rabbit and applied repeatedly to the uterus never caused a contraction, did not affect the tone of the preparation, and did not significantly alter its response to oxytocin solution (Fig. 2).

Effect of infusion of oxytocin. Solutions of oxytocin 250–500 m-u./ml. in 0·9% (0·15M) NaCl solution were infused into the rabbit at a constant rate. This rate varied in different experiments according to the size of the rabbit and the sensitivity of the uterus; in most experiments the volume infused varied from 1·1 to 4·5 ml./hr. When blood was tested, oxytocic activity was detectable about 10 min after the start of the infusion, and

after a further 5–10 min the contractions of the uterus caused by its application every 5 min remained reasonably constant (Fig. 3). When the nfusion was stopped, the activity of samples declined, and, after about 10 min, could no longer be detected. When blood was tested more often than once every 5 min the responses of the uterus became irregular, and this time interval was therefore adopted in all experiments. By comparing the contractions produced by blood with those produced by different

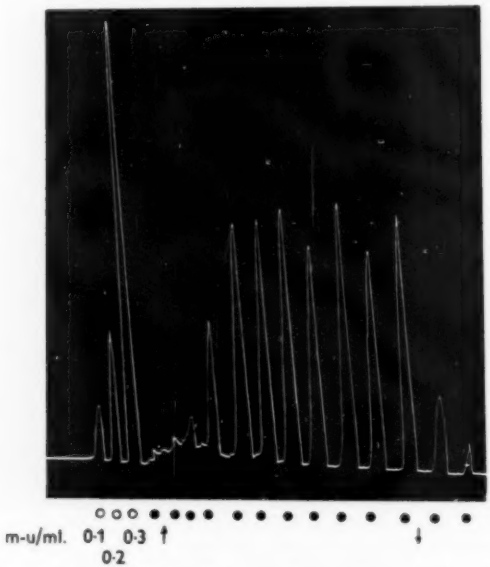

Fig. 3. Symbols as in Fig. 2. Between the arrows oxytocin was infused into the rabbit at a rate of 32·5 m-u./min.

concentrations of oxytocin it was possible to obtain rough estimates of the concentration of oxytocin in the blood. The results of five such experiments are given in Table 1.

Intracarotid saline. When 3 % (0·51 M) NaCl solution at body temperature was injected in a volume of 0·25–1·0 ml. into the tube in the carotid leading towards the head, the sample of blood taken 1·5 min later did not cause any contraction of the uterus. But if oxytocin was infused slowly and the rate of infusion adjusted so that samples had just measurable activity, blood taken 1·5 min after the intracarotid injection of 3 % NaCl solution almost invariably caused an increased response. Such an experiment is illustrated in Fig. 4. The changes in the heights of the contractions of the uterus compared with the mean height before the injection are given in Table 2, column 1. When samples of blood were taken earlier or later than

1·5 min after the injection increased oxytocic activity was less often detectable. 3% NaCl solution given intravenously never increased the oxytocic activity, and sometimes depressed it (Table 2, column 2).

TABLE 1. Oxytocic activity of rabbit's arterial blood during infusion of oxytocin

Expt. no.	Rate of infusion (m-u./min)	Approximate oxytocic activity (m-u./ml.)
1	14	0·16
2	65	0·06
3	131	0·09
4	131	0·25
5	131	0·32

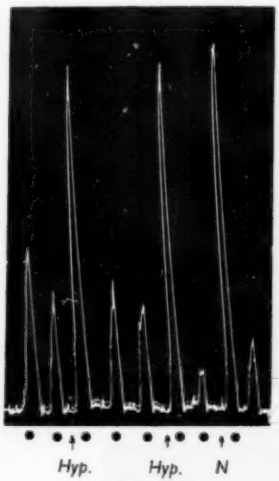

Fig. 4

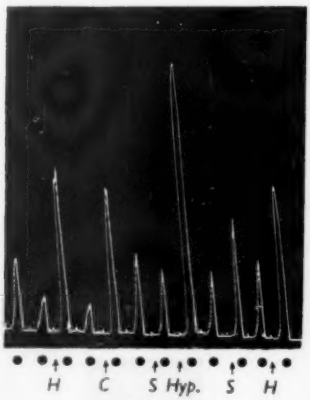

Fig. 5

Fig. 4. Samples of blood were applied (at ●) during slow infusion of oxytocin into the rabbit. Effect of intracarotid injections: *Hyp.*, 1 ml. hypertonic (3%) NaCl solution. *N*, 0·3 mg nicotine hydrogen tartrate dissolved in 1 ml. 0·9% NaCl solution.

Fig. 5. Effect of intracarotid injections, 0·5 ml. in vol. *H*, Hot 0·9% NaCl (60° C). *C*, Cold 0·9% NaCl (0° C). *Hyp.*, 3% NaCl at 37° C. *S*, 0·9% NaCl at 37° C.

When 0·9% (0·15 M) NaCl solution at body temperature was injected into the carotid artery, the response was variable, though usually there was an increase in the height of contraction (column 3). When however the same solution was heated to 60° C its injection always provoked a response (Table 2, column 4, and Fig. 5). When cooled to 0° C it was also effective, though not significantly more so than saline solution at body temperature (column 5 and Fig. 5).

TABLE 2. Oxytocic activity of rabbit's blood taken 1·5 min after various stimuli. Figures are the differences in height of contraction of uterus in mm compared with mean height before the stimulus

3% NaCl						
Intra-carotid (I.C.)	Intra-venous	0·9% NaCl (I.C.)	Hot 0·9% NaCl (I.C.)	Cold 0·9% NaCl (I.C.)	Nicotine 0·25–5 mg (I.C.)	Vagal stimulation
+65	−1	+10	+47	+12	+20	+50
+90	−10	+4	+38	+15	+35	+27
+50	−19	+1	+14	+32	+5	+30
+43	−15	+10	+50	+8	+13	−15*
+50	−8	+12	+6	—	+47	+15
+80	0	+32	+27	—	+40	−5*
+25	+1	+17	+20	—	+42	−2*
+6	—	+31	+30	—	+54	−1*
+46	—	−1	—	—	+67	+35
+50	—	+5	—	—	+35	−10*
+50	—	−2	—	—	+75	+32
+43	—	−3	—	—	+52	+48
+24	—	+2	—	—	+25	+2
+19	—	—	—	—	—	+3
+45	—	—	—	—	—	+16
+33	—	—	—	—	—	+60
+16	—	—	—	—	—	+70
+28	—	—	—	—	—	—
+45	—	—	—	—	—	—
+60	—	—	—	—	—	—
+60	—	—	—	—	—	—
Mean						
+44·2	−7·4	+9·0	+29·0	+16·7	+39·0	+20·9
S.E.						
±4·50	±2·95	±3·89	±5·49	±5·21	±5·64	±6·23

* In these experiments the nerve was stimulated repeatedly.

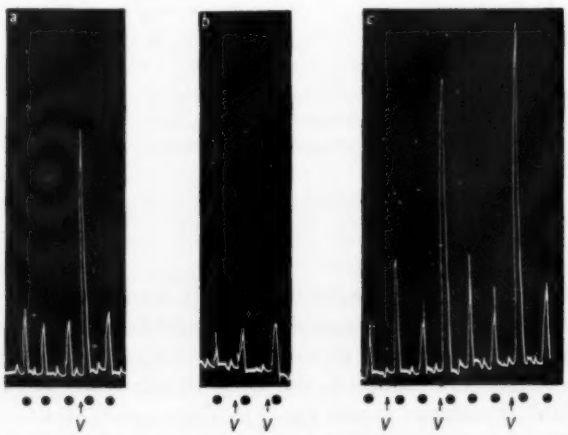

Fig. 6. *V*, Stimulation of central end of left vagus. Between (*b*) and (*c*), the preparation was left for 15 min, after which vagal stimulation was again effective.

The speed at which injections were given appeared to be an important factor. When they were rapid, about 1 ml./sec, there was a marked increase in the respiration of the animal, and an oxytocic response. If the injection was given slowly, these two effects were usually absent.

Nicotine. The intracarotid injection of 0·25–0·5 mg nicotine hydrogen tartrate always caused increased oxytocic activity in the blood taken 1·5 min later (Table 2, column 6, and Fig. 4). The injection always caused also a marked stimulation of respiration. Since control experiments have shown that the superfused rat uterus is insensitive to as much as 0·1 mg/ml. nicotine hydrogen tartrate, the increased oxytocic response was presumably not due to nicotine itself.

Fig. 7. *Hyp.*, intracarotid injection of 1 ml. 3 % saline. Between the lower arrows both jugular veins were occluded.

Stimulation of central end of left vagus. Samples of blood taken 1·5 min after stimulation of the vagus usually showed increased oxytocic activity (Table 2, column 7, and Fig. 6). In some experiments, marked in Table 2 by asterisks, the response disappeared when the nerve was stimulated repeatedly. In these experiments the response usually returned if the nerve was left unstimulated for about 15 min, or if the strength of the stimulus was increased.

Effect of clamping jugular veins. Since intracarotid injections of 3 % NaCl were effective, whereas intravenous injections were not, it seemed

likely that some oxytocic substance was being released from the head into the general circulation. If this were so, clamping the jugular veins might reduce or abolish the oxytocic effect. Accordingly, in four experiments both jugular veins were clamped from the time of the intracarotid injection until after the sample of blood had been tested. In two of these there was no oxytocic effect, and in one the effect, though detectable, was smaller than would be expected from a comparison with the effects of preceding or succeeding doses given when the jugular veins were unobstructed. In one experiment no effect was observed when blood was taken at the time when the veins were occluded, but an effect was seen when blood was tested immediately after they had been freed. This is illustrated in Fig. 7, and suggests that an oxytocic substance had accumulated in the veins beyond the clamp and was released into the general circulation when the clamps were removed.

DISCUSSION

The finding that rabbit's arterial blood can be applied directly to a superfused rat uterus without either exciting or inhibiting it was unexpected. The isolated uterus is liable to contract spontaneously unless the fluid bathing it is low in calcium and maintained at a low temperature, and it might have been expected to contract when blood at body temperature came into contact with it. In some more recent unpublished work, using a method similar to that of Vane (1958), we have found that the rat uterus can be superfused continuously with rabbit's blood without becoming spontaneously active. It may be important to avoid the use of glass tubes, which encourage the formation of oxytocic substances (Bisset & Walker, 1957), though Armstrong *et al.* (1955) found that these substances did not form in rabbit's blood.

When oxytocin was infused at a constant rate, the oxytocic activity of the arterial blood reached a fairly steady level. By comparing this activity with that of solutions of oxytocin of known strength, it was possible to estimate the concentration of oxytocin in the blood. Since, however, the sensitivity of the uterus to oxytocin in blood may well be different from its sensitivity to oxytocin in saline solution, and since it is not possible by this method to carry out a proper assay, comparisons of this sort can lead to only very approximate results. When such a calculation was done on the results of the experiment illustrated in Fig. 3, it was seen that the concentration during the infusion was about 0·25 m-u./ml. and those 5 and 10 min after stopping it were 0·14 and 0·07 m-u./ml. respectively. From this it can be calculated that the half-life of oxytocin in this animal was about 5 min, which is of the same order as the figure 3·3 min obtained by Chaudhury & Walker (1959).

During the infusion of sufficient oxytocin to give the blood just detectable oxytocic activity various stimuli caused an increased oxytocic response. Since these responses were obtained only after intracarotid injections, and since they were reduced or abolished when the jugular veins were occluded, it is likely that an oxytocic substance was released from the head. The fact that the response was most pronounced if blood was taken 1·5 min after the stimulus is consistent with this view.

Most of the stimuli used in these experiments have been previously shown to release vasopressin. Thus intracarotid hypertonic saline solution causes antidiuresis in dogs (Verney, 1947), as does intravenous nicotine in rats and in man (Burn, Truelove & Burn, 1945), and this antidiuresis is brought about by the secretion of vasopressin. Stimulation of the central end of the vagus causes secretion of vasopressin in dogs (Chang, Chia, Hsü & Lim, 1937) and produces milk ejection in goats (Andersson, 1951*b*). The effect of intracarotid hot saline solution has not been reported, though it has been shown that men exposed to high temperatures secrete antidiuretic substance in the urine (Hellman & Weiner, 1953).

The speed with which injections were given was an important factor, the effects being most marked after rapid injection. Andersson (1951*a*) also found that ejection of milk in goats only occurred if intracarotid saline was injected rapidly, at a rate of about 3 ml./sec. It is possible that baroreceptors in the carotid, which would be stimulated by our rapid injections, might play a part in producing the oxytocic response.

SUMMARY

1. A method is described in which arterial blood from a rabbit is applied at intervals to the superfused rat uterus previously soaked in dibenamine. The blood does not cause the uterus to contract, nor does it affect its subsequent response to oxytocin.

2. If oxytocin is infused into the rabbit, the blood develops oxytocic activity. When the infusion is stopped, activity rapidly decreases, and this observation can be used to calculate the approximate half-life of oxytocin.

3. If the rate of infusion is adjusted so that the blood shows just detectable oxytocic activity, any increased activity caused by certain stimuli can be detected. Intracarotid injections of 3 % NaCl, hot 0·9 % NaCl, or nicotine were effective, but intravenous 3 % NaCl was not. Intracarotid 0·9 % NaCl was sometimes effective. Stimulation of the central end of the vagus also caused an oxytocic response.

4. Reasons are given for suggesting that the results were due to a liberation of oxytocin from the neurohypophysis.

We are most grateful to Mr O. B. Saxby who designed and made the Perspex taps shown in Fig. 1 and helped us in other ways. The work was done during the tenure of a UNESCO Fellowship by S.S.

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OBSERVATIONS ON FILTER-PAPER ELECTROPHORESIS OF THYROXINE IN BARBITURATE BUFFER

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The thyroid hormone in the blood is generally assumed to be adsorbed to the serum proteins, because it is precipitated when the proteins are precipitated and cannot be dialysed from serum. If human serum containing radioactive thyroxine at physiological concentrations is submitted to paper electrophoresis in barbiturate buffer at pH 8·6, the radioactivity moves to a position between the α_1- and α_2-globulins (inter-alpha zone), whereas thyroxine alone does not move. When this observation was first made (Gordon, Gross, O'Connor & Pitt-Rivers, 1952; Larson, Deiss & Albright, 1952; Robbins & Rall, 1952; Winzler & Notrica, 1952), it was assumed that thyroxine alone fails to move in the electric field because it is adsorbed to the paper, and that it moves in the presence of serum because it is more strongly adsorbed to a substance in serum which moves to the inter-alpha position. A combination of these three lines of evidence —from protein precipitation, dialysis, and paper electrophoresis—led to the hypothesis that thyroxine is adsorbed to a specific serum protein, present in very low concentration and characterized by its electrophoretic mobility in barbiturate buffer. This hypothetical protein is usually called 'thyroxine-binding protein' (TBP), or, by some writers, 'thyroxine-binding globulin' (TBG).

It is difficult to explain the failure of thyroxine alone to move, other than by supposing that it is adsorbed to the paper, since at pH 8·6 thyroxine is negatively charged and would therefore move towards the anode if it were free to do so. However, the assumption that the movement of thyroxine in the presence of serum is due to adsorption to an inter-alpha protein seemed to us to require more definite evidence, especially since the inter-alpha zone often appears as the clearest part of the paper behind the front of the albumin band, when the paper is stained with dye to reveal the proteins. We began by studying the effect of proteolytic digestion upon the ability of serum to cause thyroxine to move electrophoretically on paper. This led to a study of certain conditions in which thyroxine moves electrophoretically in the absence of serum proteins.

METHODS

Electrophoresis. Paper electrophoresis was carried out in Perspex boxes similar to those described by Wieland (1948). The length of the bridge separating the two tanks of buffer solution was 24 cm. For electrophoresis in two dimensions, the cross-section of the box was increased so that the bridge held square sheets of filter paper (22 × 22 cm). Whatman No. 3 MM filter paper was used throughout. The radioactive thyroxine (Abbott Laboratories Pacific Ltd, Chicago), of specific activity 20–30 μc/μg, was added to the serum or other fluid at least 2 hr before the electrophoresis. The filter papers were dipped in the buffer solution, the surface moisture was allowed to drain off, and the whole sheet of paper was blotted firmly and evenly. The sample (20 μl.) was applied along a line midway between anode and cathode by spreading it along the edge of a microscope slide and then allowing the edge of the slide to touch the filter paper. Application of the sample at this position was found to increase the separation between albumin and the globulins, and to minimize the amount of movement due to the flow of water along the paper. The current was applied at 100 V and 1–4 mA for 18–24 hr at room temperature. After electrophoresis the papers were dried at 90° C, and the distribution of radioactivity was determined either by means of a continuously recording scanner or, when the electrophoresis was done in two dimensions, by making autoradiographs with Ilford X-ray film. The distribution of protein on the paper was found by staining with lissamine green and differentiating by repeated washing in 2% acetic acid. The sensitivity of this stain was such that staining was just visible when 20 μl. of a solution containing 6 mg of serum proteins/100 ml. was put on to a piece of filter paper as a single spot and then dried and stained.

Preparation of buffer. The preparation of the barbiturate buffer at pH 8·6 has already been described (Myant, 1957). Barbiturate buffers at pH 7·6 and 9·2 were made up in the same way, but the volume of 0·1 N-H_2SO_4 was adjusted to give the required pH.

Proteolytic digestion. Tryptic digestion was carried out by incubating a mixture of equal parts of serum and barbiturate buffer (pH 8·6) with 50 mg of trypsin/ml. at 37° C for 24 hr. Digestion with papain was carried out in the same way, but with the addition of a trace of NaCN to the mixture. The trypsin and papain were obtained from Hopkin and Williams Ltd.

RESULTS

Effect of proteolytic enzymes

If the ability of serum to cause thyroxine to move electrophoretically on paper is due to binding of thyroxine by one of the serum proteins, this effect should be modified or abolished by proteolytic digestion of the serum. Accordingly, observations were made on the effect of papain and trypsin upon the electrophoretic mobility of thyroxine in human serum.

Samples of serum were incubated with papain. Control samples were also incubated at the same time under similar conditions, but with no papain. At the end of the incubation, radioactive thyroxine (1 μg/100 ml.) was added to all the sera. Samples were then submitted to paper electrophoresis in barbiturate buffer at pH 8·6; papain-treated and control samples were run in parallel on separate strips of paper, so that the distribution of radioactivity in the treated serum could be correlated with the protein bands in the control serum. After electrophoresis the distribution of radioactivity was determined, and the protein bands revealed by staining.

No stain was visible on the paper on which the papain-treated serum had run, except for a narrow band at the origin in some cases. Most of the radioactivity was present as a single peak (Fig. 1*b*) with its maximum at a position corresponding to the inter-alpha zone in the control serum. The peak of activity was usually less sharp than that in the control serum, and there was often a small peak ahead of the albumin band in the control serum. The bands of protein in the control samples were similar to those seen in fresh serum, although there was some denaturation of the proteins, shown by the presence of staining between the bands. The distribution of radioactivity in the control samples (Fig. 1*a*) was the same as that normally seen in fresh serum: there was a well-defined peak in the inter-alpha zone, and a smaller peak in the albumin.

In order to test the effect of papain alone upon the electrophoretic mobility of thyroxine, radioactive thyroxine (1 μg/100 ml.) was added to a solution of papain in barbiturate buffer (50 mg/ml. buffer). Samples were then submitted to paper electrophoresis in barbiturate at pH 8·6, in parallel with samples of fresh human serum to which radioactive thyroxine had been added at the same concentration. After staining, the strip of paper to which the solution of papain had been applied showed three bands behind the line of application of the sample. The radioactivity was distributed mainly as a single peak with its maximum between the two bands nearest the origin (Fig. 1*d*). The distribution of radioactivity on the control paper was similar to that shown in Fig. 1*a*.

Similar experiments were done with trypsin instead of papain. After incubation of serum with trypsin at 37° C for 24 hr no stainable material remained on the paper after electrophoresis in the barbiturate buffer at pH 8·6. The main peak of radioactivity (Fig. 1*c*) corresponded to the inter-alpha zone, with smaller peaks near the origin and in front of the position corresponding to the albumin band. When radioactive thyroxine was incubated with trypsin alone, and the mixture separated by paper electrophoresis, most of the radioactivity moved to a position corresponding to the α_2-globulin.

The effect of pH on the mobility of thyroxine alone on paper soaked in phenylalanine (see below) led us to repeat these experiments with barbiturate buffer at pH 7·6. At this pH, radioactive thyroxine added to the control serum moved to the inter-alpha position, whereas in the papain- and trypsin-treated sera it remained at or near the line of application.

Effect of amino acids on the electrophoretic mobility of thyroxine

The results obtained with proteolytic digests of serum showed that in barbiturate buffer at pH 8·6 the distribution of thyroxine is more or less the same, whether the electrophoresis is done in the presence of normal

serum, or in the presence of serum in which the proteins have been hydrolysed. This raised the possibility that the electrophoretic mobility of thyroxine in whole serum is determined by something other than adsorption to one of the serum proteins. However, another explanation had to be considered. The binding substances might be destroyed, but the breakdown products resulting from the digestion of the serum proteins might in some way enable thyroxine to move from the line of application towards the anode during electrophoresis.

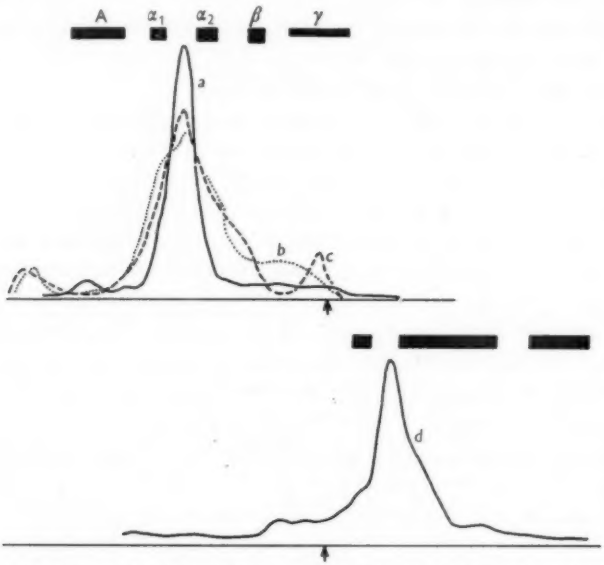

Fig. 1. Distribution of radioactivity on paper strip after electrophoresis of 20 μl. samples to which radioactive thyroxine was added (1 μg/100 ml.). *a*, normal human serum incubated at 37° C for 24 hr; *b*, the same serum digested with papain at 37° C for 24 hr; *c*, the same serum digested with trypsin; *d*, papain with no serum (50 mg/ml. of buffer). Arrows show line of application of sample. Solid blocks show positions of protein bands (A = albumin). Buffer: barbiturate at pH 8·6.

To test this possibility a mixture of pure amino acids was dissolved in water to give a solution having the same composition as a hydrolysate of 5 g of human serum albumin/100 ml. Radioactive thyroxine (1 μg/100 ml.) was added to this solution and 20 μl. samples were then submitted to paper electrophoresis in the barbiturate buffer at pH 8·6. In each experiment a sample of normal human serum containing radioactive thyroxine was run as a control on a separate strip of paper in parallel with the sample added in the solution of amino acids. After electrophoresis for 18 hr the radioactivity on the paper with the amino acids was distributed as a

single sharp peak corresponding to the inter-alpha zone on the control paper. This effect could also be obtained with mixtures of amino acids at much lower concentrations, or with solutions of single amino acids, including tyrosine, 3:5-diiodotyrosine, 3:5-diiodothyronine and tryptophane. Of all the amino acids tested, the one effective at the lowest concentration was phenylalanine. Radioactive thyroxine was dissolved in a solution of phenylalanine (1 mg/ml.) and a sample put on to a strip of paper. After electrophoresis for 18 hr the radioactivity was found to be present as a single peak at a position corresponding to the inter-alpha zone. When the paper was stained with ninhydrin, the phenylalanine could be seen as a band extending from the line of application to a line just behind the peak of radioactivity.

The same effect was obtained when radioactive thyroxine by itself was put on to a strip of paper which had been soaked along its whole length in a solution of phenylalanine (1 mg/ml.) and then allowed to dry. For this experiment two paper strips were treated by soaking them in the solution of phenylalanine, and a third strip was left untreated. The untreated paper and one of the treated papers each received radioactive thyroxine dissolved in serum. The remaining treated paper received radioactive thyroxine dissolved in barbiturate buffer. All three papers were then submitted to electrophoresis in parallel in barbiturate buffer at pH 8·6. On the treated paper with thyroxine alone, the radioactivity was distributed as a single sharp peak (Fig. 2*b*) at a position corresponding to the inter-alpha zone on the untreated paper with serum (Fig. 2*a*). Treatment of the paper with phenylalanine tended to increase the mobility of the albumin band; apart from this, the distribution of the protein bands was the same on the treated and untreated papers to which serum was added (Fig. 2*c*). On the treated paper with serum, the radioactivity was distributed partly in the inter-alpha zone and partly in the albumin, but the albumin band always carried a larger proportion of the radioactivity on the treated paper than on the untreated one (Fig. 2*a*, *c*).

The above results showed that when the paper is treated with phenylalanine, thyroxine moves electrophoretically at pH 8·6 to the inter-alpha zone in the absence of serum. This effect of phenylalanine could also be observed in the presence of human serum albumin—a protein which, when submitted to electrophoresis with thyroxine on untreated paper, carries the thyroxine to a position ahead of that corresponding to the inter-alpha zone.

Radioactive thyroxine (1 μg/100 ml. albumin solution) was dissolved in a solution of purified human serum albumin (5 g/100 ml. water) and samples, each of 20 μl., were put on to strips of filter paper previously soaked in the solution of phenylalanine. As controls, samples of the same

solution were also put on to strips of untreated filter paper. The treated and untreated paper strips were then placed side by side and submitted to electrophoresis in the barbiturate buffer at pH 8·6. After electrophoresis, about 90% of the activity on the untreated paper was present as a single peak in the albumin band (Fig. 3*a*). On the phenylalanine-treated paper, however, most of the activity was present as a broad peak corresponding to the position of the inter-alpha zone of normal human serum (Fig. 3*b*).

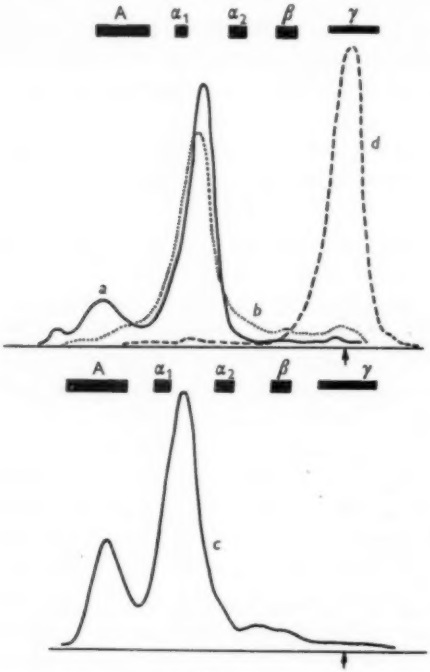

Fig 2

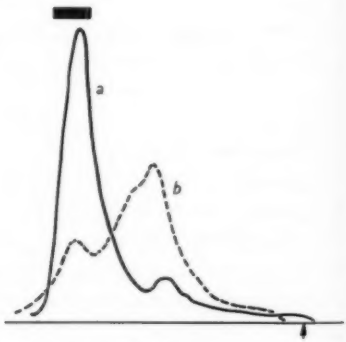

Fig. 3

Fig. 2. Distribution of radioactivity on paper-electrophoresis strip, to show effect of phenylalanine on mobility of radioactive thyroxine with and without serum. *a*, 20 μl. sample of serum run with radioactive thyroxine (1 μg/100 ml.) on untreated paper; *b*, thyroxine alone (20 μl., containing 1 μg/100 ml. of buffer) run on paper soaked in phenylalanine; *c*, same as *a*, but run on paper soaked in phenylalanine; *d*, same as *b*, but run on untreated paper. Arrows and solid blocks as in Fig. 1. Buffer: barbiturate at pH 8·6.

Fig. 3. Effect of phenylalanine on electrophoretic mobility of thyroxine in the presence of human serum albumin. *a*, distribution of radioactivity on paper strip after running 20 μl. sample of albumin solution (5 g/100 ml.) containing radioactive thyroxine (1 μg/100 ml.) on untreated paper; *b*, the same as *a*, but run on paper soaked in phenylalanine. Arrow shows line of application of samples; solid block shows position of albumin band. Buffer: barbiturate at pH 8·6.

In this paper we shall use the term 'phenylalanine effect' for the effect whereby some substances, including phenylalanine and other amino acids, enable thyroxine to move to the inter-alpha zone on paper electrophoresis in the absence of serum. The existence of the phenylalanine effect raises the possibility that the electrophoretic mobility of thyroxine in whole serum is due to the presence of the free amino acids in blood. The following experiment shows, however, that this is not so. An aqueous solution of a mixture of amino acids was made so that the concentration of each amino acid was that of the free acid in normal human serum (Stein & Moore, 1954). Radioactive thyroxine was dissolved in this solution and put on to paper strips, or was put on to paper strips which had been soaked in the amino-acid solution. After electrophoresis for 18 hr in barbiturate buffer at pH 8·6, all the radioactivity remained at the origin.

Paper electrophoresis in two dimensions

The mechanism underlying the phenylalanine effect is not known. One possibility (see Discussion) is that the phenylalanine, or other substance, blocks the binding sites on the paper which normally adsorb thyroxine, and that by doing so, it enables free thyroxine to move electrophoretically. If this is how phenylalanine acts, the serum proteins themselves might act in the same way. In this case, it would be possible to explain the movement of thyroxine to the inter-alpha zone in the presence of whole serum, without postulating a specific thyroxine-binding protein. It might be thought, for instance, that the α_1-globulin blocks enough of the binding sites in its path along the paper to enable thyroxine to move by electrophoresis to a zone just behind it. Alternatively, if the blocking effect were due to the α_2-globulin, thyroxine might be able to move just in front of it. In either case, thyroxine would travel in the inter-alpha zone. This explanation was excluded by observing the mobility of thyroxine on paper electrophoresis in two dimensions in the presence of serum.

Radioactive thyroxine was dissolved in serum (10 μg/100 ml.) and a sample of 10 μl. applied as a single spot near one corner of a square sheet of filter paper. This was submitted to electrophoresis in the barbiturate buffer. The paper was then rotated through 90° so that the serum proteins, which had been separated during the electrophoresis, lay along a line close to the edge of the paper nearest to the cathode. Electrophoresis was then carried out at the same voltage. The filter paper was dried and the positions of the radioactivity and the spots of protein were determined. The proteins were found to be distributed along a line at an angle of about 45° from the line of movement in the first dimension. The position of the radioactivitv was shown by two spots of blackening: a dark area over the inter-alpha

zone, and an area of less intense blackening over the centre of the albumin spot (Fig. 4).

The movement of thyroxine to the inter-alpha zone in the second dimension cannot have been due to the α_1- or α_2-globulin acting in the manner suggested above, since neither protein travelled along the same path as the thyroxine.

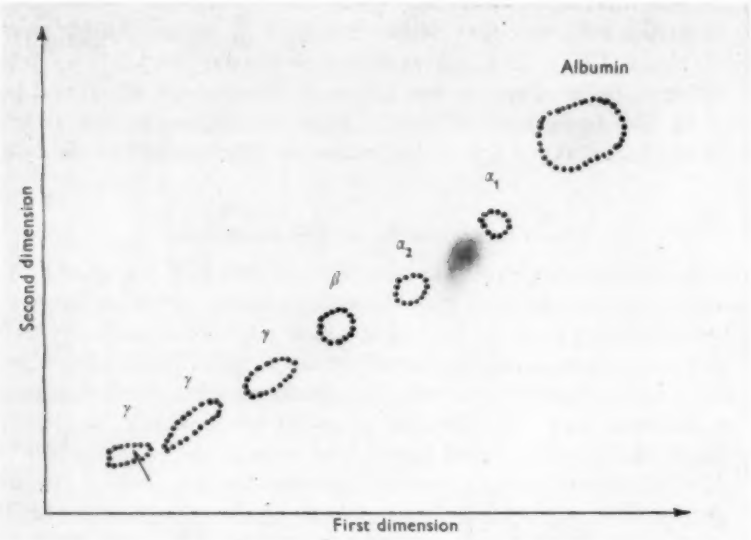

Fig. 4. Autoradiograph from two-dimensional paper electrophoresis of serum (10 μl. containing 10 μg radioactive thyroxine/100 ml.) in barbiturate buffer at pH 8·6. Arrow shows point of application of sample; areas enclosed by dotted line show positions of protein spots revealed by staining the paper; horizontal and vertical lines show direction of movement towards anode in first and second dimensions. Note: the three γ-globulin spots were observed in all two-dimensional experiments with barbiturate buffer.

Effect of pH on the electrophoretic mobility of thyroxine

If the electrophoretic mobility of thyroxine in serum is due to the presence of a substance capable of exerting the phenylalanine effect, any conditions which change the mobility of thyroxine alone on phenylalanine-treated paper should have the same effect on the mobility of thyroxine in the presence of serum. Changes in the pH of the buffer are known to influence the mobility of thyroxine submitted to paper electrophoresis in serum (Robbins, Petermann & Rall, 1955). Making use of this observation, we have compared the electrophoretic mobility of thyroxine alone run on

paper soaked in a solution of phenylalanine, with that of thyroxine dissolved in serum and run on untreated paper, using barbiturate buffer at pH 7·6, 8·6 and 9·2. In each experiment two strips of paper, one untreated and the other treated with phenylalanine, were placed in parallel in the same electrophoresis box. The untreated paper received 20 μl. of serum containing radioactive thyroxine (1 μg/100 ml.) and the phenylalanine-treated paper received 20 μl. of a solution of radioactive thyroxine in the buffer (1 μg/100 ml. buffer). At the end of the run, the

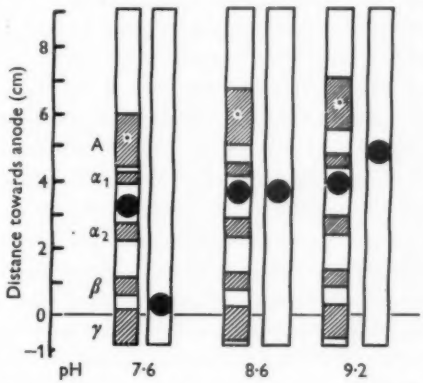

Fig. 5. Diagram to represent paper strips, showing effect of pH of barbiturate buffer on distribution of thyroxine submitted to electrophoresis with serum on untreated paper, and without serum on paper soaked in phenylalanine. The strip on the left of each pair was untreated and received radioactive thyroxine dissolved in serum (1 μg/100 ml.); that on the right of each pair was soaked in phenylalanine solution (1 mg/ml.) and received radioactive thyroxine dissolved in the buffer (1 μg/100 ml.). Shaded areas show positions of protein bands, averaged from many experiments; solid circles show positions of peaks of radioactivity, the proportion of the total activity at each peak being shown roughly by the area of the circle. Vertical scale, distance towards anode from line of application of sample; horizontal scale, pH.

distribution of radioactivity was found, and the mobility of the peaks expressed in centimetres per hour. The papers were then stained, so that the positions of the peaks of activity could be seen in relation to the bands of serum protein. Preliminary experiments showed that thyroxine applied to untreated paper without serum did not move more than a few millimetres when submitted to electrophoresis in any of the conditions to be tested.

The results obtained at pH 8·6 have been described in the preceding section (Figs. 2, 5).

At pH 7·6, on the untreated paper with thyroxine dissolved in serum, the mobility of all the serum proteins was less than at pH 8·6. The reduction

in mobility was most marked in the case of the albumin band, which sometimes merged with the α_1-globulin. The mobility of the main peak of radioactivity was also reduced, but its relationship to the α_1- and α_2-globulins remained unchanged (Fig. 5). The size of the small peak of activity in the albumin was about the same as with barbiturate buffer at pH 8·6. On the treated paper, to which the radioactive thyroxine had been added without serum, the radioactivity usually remained at the line of application. In a few experiments it moved as a single peak a little towards the anode, but never beyond a position corresponding to the β-globulin band on the control paper containing serum.

At pH 9·2 all the serum protein fractions moved further than at pH 8·6. The increase in mobility was most marked in the case of albumin, and this resulted in a widening of the gap between albumin and the α_1-globulin. The main peak of radioactivity on the paper with serum was further towards the anode than at pH 8·6 (Fig. 5), but its relationship to the α_1- and α_2-globulins was the same. On the phenylalanine-treated paper the radioactivity was distributed as a single peak ahead of the main peak of activity on the untreated paper to which the thyroxine had been added in serum. In some experiments the peak of activity on the treated paper corresponded to the position of the α_1-globulin on the untreated paper; in others it was between the albumin and α_1-globulin bands (Fig. 5).

DISCUSSION

The simplest way of explaining our observations is to suppose that in the presence of certain substances which compete for thyroxine-binding sites on the paper, thyroxine moves electrophoretically as the free molecule, and that free thyroxine moves on paper to the inter-alpha zone in barbiturate buffer at pH 8·6 but not at pH 7·6 or 9·2. If phenylalanine competes for the thyroxine-binding sites on albumin, as well as for those on the paper, this would explain why thyroxine moves to the inter-alpha zone on phenylalanine-treated paper in the presence of serum albumin (Fig. 3).

The electrophoretic movement of thyroxine in serum cannot be due to the phenylalanine effect, since thyroxine in the presence of whole serum continues to move to the inter-alpha zone when the pH is changed from 8·6 to 7·6 or 9·2, whereas it does not do so when run alone on phenylalanine-treated paper (Fig. 5). To this extent our observations give some evidence in favour of the view that the electrophoretic mobility of thyroxine in serum is determined by that of a substance to which the thyroxine is bound. The presence of a binding substance with a limited capacity also explains the well-known observation that only a limited amount of thyroxine can be carried in the inter-alpha zone—an effect that is difficult to account for in any other way. In the presence of serum that has been

digested by proteolytic enzymes, thyroxine has a mobility similar to that of thyroxine on phenylalanine-treated paper in the absence of serum—that is, it moves to the inter-alpha zone at pH 8·6 but not at pH 7·6. This is consistent with the current view that thyroxine in serum is bound to a specific protein.

There is no direct evidence that phenylalanine and other substances which enable thyroxine to move electrophoretically in the absence of serum act in the manner suggested above. It is possible that they act by forming complexes with thyroxine, although this would raise the difficulty of explaining why complexes formed between thyroxine and different amino acids all have the same electrophoretic mobility in barbiturate buffer. It must also be pointed out that if the binding of thyroxine by filter paper is completely suppressed by phenylalanine, thyroxine would be expected to move towards the anode on phenylalanine-treated paper at pH 7·6, since this pH is higher than the iso-electric point of thyroxine. It may be that there is, in fact, some tendency for thyroxine to move towards the anode at pH 7·6, and that this is balanced by a flow of buffer towards the cathode. It is also possible that the binding of thyroxine by the paper is only partially suppressed by substances which exert the phenylalanine effect; we have been unable to show that treatment with phenylalanine has any effect on the ease with which thyroxine can be eluted from a piece of filter paper.

Phenylalanine does not act by chelating traces of heavy metals in the filter paper, since thyroxine remains at the origin after electrophoresis at pH 8·6 on filter paper soaked in ethylenediaminetetraacetic acid (Osorio & Myant, unpublished).

Whatever the true explanation for the phenylalanine effect may be, the results obtained with proteolytic enzymes and with phenylalanine show that, under certain conditions, thyroxine may behave electrophoretically in the absence of serum as if a specific binding substance were present. This complicates the problem of testing for the presence of the binding substance, presumed to be present in whole serum, when attempting to purify it or to demonstrate its presence in body fluids other than serum. Demonstration, for example, that an extract of serum or other tissue causes thyroxine to move to the inter-alpha zone, when submitted to paper electrophoresis in barbiturate buffer at pH 8·6 in the presence of albumin, does not prove that the extract contains the substance in whole serum which causes thyroxine to move to the inter-alpha zone under these conditions ('inter-alpha binding substance').

This raises the question as to how the 'inter-alpha binding substance' should be defined. We suggest that it should be defined at present as a substance in human serum which, when tested by filter-paper electrophoresis

in barbiturate buffer over a range of pH from 7·6 to 9·2 and of ionic strength from 0·05 to 0·1, carries thyroxine at physiological concentrations to the inter-alpha zone, both in the presence and in the absence of serum; when added to normal serum it increases the thyroxine-binding capacity at the inter-alpha zone.

The binding substance, it may be noted, is defined here solely in terms of its association with thyroxine and its electrophoretic behaviour on paper under specified conditions. An association between thyroxine and specific fractions of human serum may be observed under other conditions; as by ultracentrifugation (Petermann, Robbins & Hamilton, 1954), electrophoresis in the Tiselius apparatus (Silver & Reiner, 1950), electrophoresis with starch gel as the supporting medium (Rich & Bearn, 1958), separation of serum proteins by chromatography with ion-exchange resins (Ingbar, Dowling & Freinkel, 1957), or determination of the iodine content of proteins obtained by fractionation of whole serum (Bassett, Coons & Salter, 1941; Schmid, 1953). However, it is not certain that any of the binding substances revealed by these methods is the same as the inter-alpha substance defined above. This uncertainty arises partly because the interaction between thyroxine and the binding substances in serum is reversible and may be influenced by the method used for observing it. On paper electrophoresis, for instance, the distribution of thyroxine is influenced by the pH and composition of the buffer (Myant & Osorio, 1960). Other factors, such as the supporting medium used in zone electrophoresis, or the conditions used in the Cohn fractionation, may well have an equally marked effect on the distribution of thyroxine in serum.

SUMMARY

1. When thyroxine is submitted to paper electrophoresis in the presence either of a proteolytic digest of human serum or of phenylalanine or other amino acids, it moves in a barbiturate buffer at pH 8·6 to a position corresponding to the inter-alpha zone. Under the same conditions, thyroxine scarcely moves from the origin at pH 7·6. The term 'phenylalanine effect' is proposed for this effect.

2. The phenylalanine effect may be explained by the hypothesis that certain substances, including phenylalanine and substances present in a proteolytic digest of serum, enable thyroxine to move electrophoretically as the free molecule by blocking the thyroxine-binding sites on the paper, and that free thyroxine moves on paper at pH 8·6 but not at pH 7·6.

3. Since thyroxine in the presence of whole serum moves to the inter-alpha zone in barbiturate buffer at pH 7·6, it is concluded that the electrophoretic mobility of thyroxine in serum is not due to the phenyl-

alanine effect. This is taken as evidence for the existence of a specific thyroxine-binding substance in serum.

4. The behaviour of thyroxine in serum submitted to paper electrophoresis in two dimensions is not consistent with the hypothesis that either the α_1- or the α_2-globulin causes thyroxine to move electrophoretically by exerting the phenylalanine effect.

5. It is pointed out that the existence of the phenylalanine effect complicates the problem of testing for the substance presumed to be responsible for the electrophoretic mobility of thyroxine in whole serum in barbiturate buffer, since thyroxine alone may behave as if a binding substance were present.

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